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(54) Title: HERBICIDE TARGET GENES AND METHODS

(57) Abstract: The present invention provides plant ENR-A, CBL, UROD, PBGD, and CPPO genes. Also disclosed are the recombinant production of ENR-A, CBL, UROD, PBGD and CPPO enzymes in heterologous hosts, screening chemicals for herbicidal activity using these recombinantly produced enzymes, and the use of thereby identified herbicidal chemicals to suppress the growth of undesired vegetation. Furthermore, the present invention provides methods for the development of herbicide tolerance in plants, plant tissues, plant seeds, and plant cells using ENR-A, CBL, UROD, PBGD, and CPPO genes of the invention.

HERBICIDE TARGET GENES AND METHODS

The invention relates generally to enzymatic activity involved in ENR-A, CBL, UROD, PBGD, or CPPO in plants. In particular, the invention relates to plant genes that encode a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity. The invention has various utilities, including the recombinant production of polypeptides having ENR-A, CBL, UROD, PBGD, or CPPO activity in heterologous hosts, the screening of chemicals for herbicidal activity, and the use of thereby identified herbicidal chemicals to control the growth of undesired vegetation. The invention may also be applied to the development of herbicide tolerance in plants, plant tissues, plant seeds, and plant cells.

The use of herbicides to control undesirable vegetation such as weeds in crop fields has become almost a universal practice. The herbicide market exceeds 15 billion dollars annually. Despite this extensive use, weed control remains a significant and costly problem for farmers.

For example, present herbicides often impose special limitations on farming practices, and the time and method of application and stage of weed plant development often are critical for good weed control with such herbicides, thus creating farm management constraints. Furthermore, since only a few target enzymes are inhibited by currently used herbicides, various weed species are, or may become, resistant to these herbicides. For all of these reasons, the discovery and development of effective new herbicides, in particular those acting on novel target enzymes, is increasingly important.

Novel herbicides can now be discovered using high-throughput screens that implement recombinant DNA technology. Once identified, metabolic enzymes essential to plant growth and development can be recombinantly produced through standard molecular biological techniques and utilized as herbicide targets in screens for novel inhibitors of the enzyme's activity. The novel inhibitors discovered through such screens may then be used as herbicides to control undesirable vegetation. Such herbicides are also useful for selecting herbicide tolerant plants, and seed plants tolerant to the herbicide can be produced, for example by genetic engineering techniques. Thus, herbicides that exhibit greater potency, broader weed spectrum, and more rapid degradation in soil can be applied to crops that are resistant or tolerant to herbicides in order to kill weeds without attendant risk of damage to the crop.

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Therefore, in order to meet the future food requirements of the world's growing population in a cost-effective and environmentally safe manner, there exists a long felt and unfulfilled need for novel target enzymes for herbicides, for new and better herbicides inhibiting such target enzymes and for plants tolerant to these new and better herbicides.

The present invention thus provides:

isolated DNA molecules comprising

a nucleotide sequence encoding an amino acid sequence substantially similar to SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:10, but particularly to SEQ ID NO:6 or SEQ ID NO:10

a nucleotide sequence substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9, but particularly to SEQ ID NO:5, or SEQ ID NO:9

in particular

- wherein said nucleotide sequence is a plant nucleotide sequence
- wherein the amino acid sequence has ENR-A, CBL, UROD, PBGD, or CPPO activity, but particularly UROD or CPPO activity

The present invention further provides a polypeptide comprising an amino acid sequence encoded by a nucleotide sequence substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9, in particular wherein

- the polypeptide comprises an amino acid sequence encoded by a nucleotide sequence substantially similar to SEQ ID NO:5 or SEQ ID NO:9
- said amino acid sequence is substantially similar to SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:10
- said amino acid sequence is substantially similar to SEQ ID NO:6 or SEQ ID NO:10
- said amino acid sequence has ENR-A, CBL, UROD, PBGD, or CPPO activity, but particularly UROD or CPPO activity

Further comprised by the present invention are polypeptides comprising an amino acid sequence comprising at least 20, particularly 50 and more particularly 100 consecutive amino acid residues of the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8 or SEQ ID NO:10, but particularly of SEQ ID NO:6 or SEQ ID NO:10.

Further provided are

- expression cassettes comprising a promoter operatively linked to a DNA molecule according to the invention and recombinant vectors comprising said expression cassettes

- host cells comprising a DNA molecule according to the invention, in particular, wherein said host cell is selected from the group consisting of an insect cell, a yeast cell
- prokaryotic cells and plant cells, plants or seeds comprising a plant cell according to the invention
- plants according to the invention, wherein said plants are tolerant to an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity, but particularly to an inhibitor of UROD or CPPO activity

Further provided are

- expression cassettes comprising a promoter functional in a eukaryote operatively linked to a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9, but particularly to SEQ ID NO:1 or SEQ ID NO:7
- recombinant vectors comprising said expression cassettes
- host cells comprising said expression cassette, in particular wherein said host cells are selected from the group consisting of insect cells, yeast cells, prokaryotic cells and plant cells
- plant cells comprising an isolated DNA molecule comprising a nucleotide sequence identical or substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9, but particularly to SEQ ID NO:1 or SEQ ID NO:7
- plants or seed comprising a plant cell of the invention
- plants according to the invention, wherein said plants are tolerant to an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity, but particularly to an inhibitor of ENR-A or PBGD activity
- host cells comprising an expression cassette, comprising a promoter operatively linked to an isolated DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:3, wherein said host cell is an eukaryotic cell
- a host cell as mentioned hereinbefore, wherein said host cell is selected from the group consisting of an insect cell, a yeast cell, and a plant cell
- a plant or seed comprising a plant cell as mentioned hereinbefore
- a plant according to the invention, wherein said plant is tolerant to an inhibitor of CBL activity

Further provided are methods comprising:

- a) combining a polypeptide comprising the amino acid sequence encoded by a nucleotide sequence substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7,

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or SEQ ID NO:9, or a homolog thereof, and a compound to be tested for the ability to interact with said polypeptide, under conditions conducive to interaction; and
b) selecting a compound identified in step (a) that is capable of interacting with said polypeptide and, optionally, further comprising:
c) applying a compound selected in step (b) to a plant to test for herbicidal activity; and
d) selecting compounds having herbicidal activity.

Also provided are compounds identifiable by a method according to the invention, in particular compounds having herbicidal activity.

Further provided is a process of identifying an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity comprising:

- a) introducing a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, and encoding a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity, or a homolog thereof, into a plant cell, such that said sequence is functionally expressible at levels that are higher than wild-type expression levels;
- b) combining said plant cell with a compound to be tested for the ability to inhibit the ENR-A, CBL, UROD, PBGD, or CPPO activity under conditions conducive to such inhibition;
- c) measuring plant cell growth under the conditions of step (b);
- d) comparing the growth of said plant cell with the growth of a plant cell having unaltered ENR-A, CBL, UROD, PBGD, or CPPO activity under identical conditions; and
- e) selecting said compound that inhibits plant cell growth in step (d)

and compounds having herbicidal activity identifiable according to the process of the invention.

In view of these long felt yet unfulfilled needs, one object of the invention is to provide a method for identifying new or improved herbicides. Another object of the invention is to provide a method for using such new or improved herbicides to suppress the growth of plants such as weeds. Still another object of the invention is to provide improved crop plants, and seed thereof, that are tolerant to such new or improved herbicides.

In furtherance of these and other objects, the present invention provides DNA molecules comprising a nucleotide sequence, preferably isolated from a plant, that encode a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity. The inventors are the first to demonstrate that the ENR-A, CBL, UROD, PBGD, or CPPO genes are essential for the growth of a plant, and therefore are good target enzymes for identifying new herbicides.

According to one embodiment, the present invention provides a DNA molecule comprising a nucleotide sequence isolated from a plant that encodes the polypeptide set forth in any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. For example, the DNA molecule of the invention may comprise a nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, respectively. In another example, the DNA molecule of the invention comprises a nucleotide sequence that is substantially similar to any one of the coding sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, and that encodes a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively. Although a nucleotide sequence provided in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, is isolated from *Arabidopsis thaliana*, using the information provided by the present invention, other nucleotide sequences that encode a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity are obtained from other sources, e.g. from other plants, using standard methods known in the art.

The present invention also provides a nucleotide sequence construct comprising a promoter operatively linked to a DNA molecule of the invention. Further, the present invention provides methods to stably transform such a nucleotide sequence construct into a host cell, and host cells comprising such a nucleotide sequence construct, wherein the host cell is capable of expressing the DNA molecule encoding a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively. Any suitable cell may be used as a host cell, e.g. a bacterial cell, a yeast cell, or a plant cell.

In accordance with another embodiment, the present invention also relates to the recombinant production of a ENR-A, CBL, UROD, PBGD, or CPPO polypeptide and methods of use of ENR-A, CBL, UROD, PBGD, or CPPO in assays for identifying compounds that interact with ENR-A, CBL, UROD, PBGD, or CPPO polypeptide, respectively. In a preferred embodiment, the present invention provides a plant polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity useful for identifying inhibitors of ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, in *in vivo* and *in vitro* assays. Preferably the isolated polypeptide of the present invention comprises an amino acid sequence substantially similar to any one of the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10, respectively. More preferably, this enzyme comprises the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10.

The present invention further provides methods of using purified polypeptides having ENR-A, CBL, UROD, PBGD, or CPPO activity, preferably polypeptides derived from plant sources, in assays to screen for and identify compounds that interact with a ENR-A, CBL, UROD, PBGD, or CPPO polypeptide, respectively. Such compounds are preferably inhibitors of ENR-A, CBL, UROD, PBGD, or CPPO activity, and are potentially herbicides of future commercial interest. The inhibitors are used as herbicides to suppress the growth of undesirable vegetation in fields where crops are grown, particularly agronomically important crops such as maize and other cereal crops such as wheat, oats, rye, sorghum, rice, barley, millet, turf and forage grasses, and the like, as well as cotton, sugar cane, sugar beet, oilseed rape, and soybeans.

Thus, an assay useful for identifying inhibitors of essential plant genes, such as plant ENR-A, CBL, UROD, PBGD, or CPPO genes, comprises the steps of:

- a) reacting a plant ENR-A, CBL, UROD, PBGD, or CPPO enzyme, and a substrate thereof in the presence of a suspected inhibitor of the enzyme's function;
- b) comparing the rate of enzymatic activity in the presence of the suspected inhibitor to the rate of enzymatic activity under the same conditions in the absence of the suspected inhibitor; and
- c) determining whether the suspected inhibitor inhibits the ENR-A, CBL, UROD, PBGD, or CPPO enzyme, respectively.

For example, the inhibitory effect on plant ENR-A, CBL, UROD, PBGD, or CPPO may be determined by a reduction or complete inhibition of ENR-A, CBL, UROD, PBGD, or CPPO activity in the assay. Such a determination may be made by comparing, in the presence and absence of the candidate inhibitor, the amount of substrate used or intermediate or product made during the reaction.

The present invention further embodies plants, plant tissues, plant seeds, and plant cells that have modified ENR-A, CBL, UROD, PBGD, or CPPO activity, and that are therefore tolerant to inhibition by a chemical at levels normally inhibitory to naturally occurring ENR-A, CBL, UROD, PBGD, or CPPO enzyme activity, respectively. Herbicide tolerant plants encompassed by the invention include those that would otherwise be potential targets for normally inhibiting herbicides, particularly the agronomically important crops mentioned above. According to one aspect of this embodiment, plants, plant tissue, plant seeds, or plant cells are stably transformed with a recombinant DNA molecule comprising a suitable promoter functional in plants operatively linked to a nucleotide sequence that encodes an

enzyme having modified ENR-A, CBL, UROD, PBGD, or CPPO activity, that is tolerant to a concentration of a ENR-A, CBL, UROD, PBGD, or CPPO inhibitor, respectively, that would normally inhibit the activity of wild-type, unmodified ENR-A, CBL, UROD, PBGD, or CPPO, in the plant. Modified ENR-A, CBL, UROD, PBGD, or CPPO activity, may also be conferred upon a plant by increasing expression of wild-type (i.e. sensitive) ENR-A, CBL, UROD, PBGD, or CPPO enzyme, by providing multiple copies of wild-type ENR-A, CBL, UROD, PBGD, or CPPO genes, to the plant or by overexpression of the endogenous wild-type ENR-A, CBL, UROD, PBGD, or CPPO gene, or genes, under control of a stronger-than-wild-type promoter, e.g., either a promoter that drives expression at a higher rate, or a promoter that drives expression for a longer duration. The transgenic plants, plant tissue, plant seeds, or plant cells thus created are then selected by conventional selection techniques, whereby inhibitor tolerant descendants (lines) are isolated, characterized, and developed. Alternately, random or site-specific mutagenesis may be used to generate ENR-A, CBL, UROD, PBGD, or CPPO inhibitor tolerant lines. Still further, inhibitor tolerant lines can be developed via selection of natural variants.

Therefore, the present invention provides a plant, plant cell, plant seed, or plant tissue comprising a DNA molecule comprising a nucleotide sequence, preferably isolated from a plant, that encodes an enzyme having ENR-A, CBL, UROD, PBGD, or CPPO activity, and wherein the DNA molecule confers upon the plant, plant cell, plant seed, or plant tissue tolerance to a ENR-A, CBL, UROD, PBGD, or CPPO inhibitor, in amounts that normally naturally occurring ENR-A, CBL, UROD, PBGD, or CPPO activity. According to one example of this embodiment, the enzyme comprises an amino acid sequence substantially similar to any one of the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. According to another example of this embodiment, the DNA molecule is substantially similar to any one of the coding sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9. In a related aspect, the present invention is directed to a method for selectively suppressing the growth of weeds in a field containing a crop of planted crop seeds or plants, comprising applying to crops or crop seeds that are tolerant to an inhibitor that inhibits naturally occurring ENR-A, CBL, UROD, PBGD, or CPPO activity, and the weeds in the field an ENR-A, CBL, UROD, PBGD, or CPPO inhibitor, respectively, in amounts that inhibit naturally occurring ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, wherein the inhibitor suppresses the growth of the weeds without significantly suppressing the growth of the crops.

Other objects and advantages of the present invention will become apparent to those skilled in the art from a study of the following description of the invention and non-limiting examples.

The invention thus provides:

An isolated DNA molecule comprising a nucleotide sequence substantially similar to any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9. In a preferred embodiment, the nucleotide sequence encodes an amino acid sequence substantially similar to any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. In another preferred embodiment, the nucleotide sequence is SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9. In yet another preferred embodiment, the nucleotide sequence encodes the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. Preferably, the nucleotide sequence is a plant nucleotide sequence, which preferably encodes a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity.

The invention further provides:

A polypeptide comprising an amino acid sequence encoded by a nucleotide sequence substantially similar to any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9. Preferably, the amino acid sequence is encoded by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9. Preferably, the polypeptide comprises an amino acid sequence substantially similar to any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. Preferably the amino acid sequence is SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. The amino acid sequence preferably has ENR-A, CBL, UROD, PBGD, or CPPO activity. In another preferred embodiment, the amino acid sequence comprises at least 20 consecutive amino acid residues of the amino acid sequence encoded by any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9. Or, alternatively, the amino acid sequence comprises at least 20 consecutive amino acid residues of the amino acid sequence of any one of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10.

The invention further provides:

An expression cassette comprising a promoter operatively linked to a DNA molecule according to the present invention, wherein the promoter is preferably functional in a

eukaryote, wherein the promoter is preferably heterologous to the DNA molecule. The present invention further provides a recombinant vector comprising an expression cassette according to the present invention, wherein said vector is preferably capable of being stably transformed into a host cell, a host cell comprising a DNA molecule according to the present invention, wherein said DNA molecule is preferably expressible in the cell. The host cell is preferably selected from the group consisting of an insect cell, a yeast cell, a prokaryotic cell and a plant cell. The invention further provides a plant or seed comprising a plant cell of the present invention, wherein the plant or seed is preferably tolerant to an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity.

The invention further provides:

A process for making nucleotides sequences encoding gene products having altered ENR-A, CBL, UROD, PBGD, or CPPO activity, comprising: a) shuffling an unmodified nucleotide sequence of the present invention, b) expressing the resulting shuffled nucleotide sequences, and c) selecting for altered ENR-A, CBL, UROD, PBGD, or CPPO activity, as compared to the ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, of the gene product of said unmodified nucleotide sequence.

In a preferred embodiment, the unmodified nucleotide sequence is identical or substantially similar to any one of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, or a homolog thereof. The present invention further provides a DNA molecule comprising a shuffled nucleotide sequence obtainable by the process described above, a DNA molecule comprising a shuffled nucleotide sequence produced by the process described above. Preferably, a shuffled nucleotide sequence obtained by the process described above has enhanced tolerance to an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity. The invention further provides an expression cassette comprising a promoter operatively linked to a DNA molecule comprising a shuffled nucleotide sequence a recombinant vector comprising such an expression cassette, wherein said vector is preferably capable of being stably transformed into a host cell, a host cell comprising such an expression cassette, wherein said nucleotide sequence is preferably expressible in said cell. A preferred host cell is selected from the group consisting of an insect cell, a yeast cell, a prokaryotic cell and a plant cell. The invention further provides a plant or seed comprising such plant cell, wherein the plant is preferably tolerant to an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively.

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The invention further provides:

A method for selecting compounds that interact with the protein encoded by SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, comprising: a) expressing a DNA molecule comprising SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, or a sequence substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, or a homolog thereof, to generate the corresponding protein, b) testing a compound suspected of having the ability to interact with the protein expressed in step (a), and c) selecting compounds that interact with the protein in step (b).

The invention further provides:

A process of identifying an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity, comprising: a) introducing a DNA molecule comprising a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, and having ENR-A, CBL, UROD, PBGD, or CPPO activity, or nucleotide sequences substantially similar thereto, or a homolog thereof, into a plant cell, such that said sequence is functionally expressible at levels that are higher than wild-type expression levels, b) combining said plant cell with a compound to be tested for the ability to inhibit the ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, under conditions conducive to such inhibition, c) measuring plant cell growth under the conditions of step (b),

d) comparing the growth of said plant cell with the growth of a plant cell having unaltered ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, under identical conditions, and e) selecting said compound that inhibits plant cell growth in step (d).

The invention further comprises a compound having herbicidal activity identifiable according to the process described immediately above.

The invention further comprises:

A process of identifying compounds having herbicidal activity comprising:

a) combining a protein of the present invention and a compound to be tested for the ability to interact with said protein, under conditions conducive to interaction, b) selecting a compound identified in step (a) that is capable of interacting with said protein, c) applying identified compound in step (b) to a plant to test for herbicidal activity, and d) selecting compounds having herbicidal activity.

The invention further comprises a compound having herbicidal activity identifiable according to the process described immediately above.

The invention further comprises:

A method for suppressing the growth of a plant comprising, applying to said plant a compound that inhibits the activity of a polypeptide of the present invention in an amount sufficient to suppress the growth of said plant.

The invention further comprises:

A method for recombinantly expressing a protein having ENR-A, CBL, UROD, PBGD, or CPPO activity comprising introducing a nucleotide sequence encoding a protein having one of the above activities into a host cell and expressing the nucleotide sequence in the host cell. A preferred host cell is selected from the group consisting of an insect cell, a yeast cell, a prokaryotic cell and a plant cell. A preferred prokaryotic cell is a bacterial cell, e.g. *E. coli*.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1 is a cDNA sequence encoding ENR-A from *Arabidopsis thaliana*.

SEQ ID NO:2 is the predicted amino acid sequence of *Arabidopsis thaliana* ENR-A encoded by SEQ ID NO:1.

SEQ ID NO:3 is cDNA coding sequence for the CBL gene from *Arabidopsis thaliana*.

SEQ ID NO:4 amino acid sequence encoded by the *Arabidopsis thaliana* CBL sequence shown in SEQ ID NO:3.

SEQ ID NO:5 is a cDNA sequence encoding UROD from *Arabidopsis thaliana*.

SEQ ID NO:6 is the predicted amino acid sequence of *Arabidopsis thaliana* UROD encoded by SEQ ID NO:5.

SEQ ID NO:7 is a cDNA sequence encoding PBGD from *Arabidopsis thaliana*.

SEQ ID NO:8 is the predicted amino acid sequence of *Arabidopsis thaliana* PBGD encoded by SEQ ID NO:7.

SEQ ID NO:9 is a cDNA sequence encoding CPPO from *Arabidopsis thaliana*.

SEQ ID NO:10 is the predicted amino acid sequence of *Arabidopsis thaliana* CPPO encoded by SEQ ID NO:9.

SEQ ID NO:11 is the genomic sequence of the ENR-A gene from *Arabidopsis thaliana*.

SEQ ID NO:12 is the oligonucleotide ENR-A-F2

SEQ ID NO:13 is the oligonucleotide ENR-A-R2

SEQ ID NO:14 is the sequence for oligonucleotide DG354.

SEQ ID NO:15 is the sequence for oligonucleotide DG357.

SEQ ID NO:16 is the sequence for oligonucleotide CBL1.
SEQ ID NO:17 is the sequence for oligonucleotide CBL2.
SEQ ID NO:18 is the sequence for oligonucleotide CBL3.
SEQ ID NO:19 is the sequence for oligonucleotide ASV1.
SEQ ID NO:20 is the sequence for oligonucleotide ASV2.
SEQ ID NO:21 is the genomic sequence of *Arabidopsis thaliana* UROD.
SEQ ID NO:22 is the sequence for oligonucleotide UROD-N-Nde.
SEQ ID NO:23 is the sequence for oligonucleotide UROD-C-Not.
SEQ ID NO:24 is the sequence for oligonucleotide UROD-F2.
SEQ ID NO:25 is the sequence for oligonucleotide UROD-R2.
SEQ ID NO:26 is the genomic sequence of *Arabidopsis thaliana* PBGD.
SEQ ID NO:27 is the sequence for oligonucleotide PORD-F2.
SEQ ID NO:28 is the sequence for oligonucleotide PORD-R2.
SEQ ID NO:29 is the genomic sequence of the CPPO gene from *Arabidopsis thaliana*.
SEQ ID NO:30 is the sequence for oligonucleotide CR73.
SEQ ID NO:31 is the sequence for oligonucleotide CR75.
SEQ ID NO:32 is the sequence for oligonucleotide JG-L.
SEQ ID NO:33 is the sequence for oligonucleotide CPPGO-F2.
SEQ ID NO:34 is the sequence for oligonucleotide CPPGO-R2.

For clarity, certain terms used in the specification are defined and used as follows:

Activatable DNA Sequence: a DNA sequence that regulates the expression of genes in a genome, desirably the genome of a plant. The activatable DNA sequence is complementary to a target gene endogenous in the genome, in this case the gene encoding ENR-A, CBL, UROD, PBGD, or CPPO. When the activatable DNA sequence is introduced and expressed in a cell, it inhibits expression of the target gene. An activatable DNA sequence useful in conjunction with the present invention includes those encoding or acting as dominant inhibitors, such as a translatable or untranslatable sense sequence capable of disrupting gene function in stably transformed plants to positively identify one or more genes essential for normal growth and development of a plant. A preferred activatable DNA sequence is an antisense DNA sequence. The interaction of the antisense sequence and the target gene results in substantial inhibition of the expression of the target gene so as to kill the plant, or at least inhibit normal plant growth or development.

Activatable DNA Construct: a recombinant DNA construct comprising a synthetic promoter operatively linked to the activatable DNA sequence, which when introduced into a cell, desirably a plant cell, is not expressed, i.e. is silent, unless a complete hybrid transcription factor capable of binding to and activating the synthetic promoter is present. The activatable DNA construct is introduced into cells, tissues, or plants to form stable transgenic lines capable of expressing the activatable DNA sequence.

Antiparallel: "Antiparallel" refers herein to two nucleotide sequences paired through hydrogen bonds between complementary base residues with phosphodiester bonds running in the 5'-3' direction in one nucleotide sequence and in the 3'-5' direction in the other nucleotide sequence.

Co-factor: natural reactant, such as an organic molecule or a metal ion, required in an enzyme-catalyzed reaction. A co-factor is e.g. NAD(P), riboflavin (including FAD and FMN), folate, molybdopterin, thiamin, biotin, lipoic acid, pantothenic acid and coenzyme A, S-adenosylmethionine, pyridoxal phosphate, ubiquinone, menaquinone. Optionally, a co-factor can be regenerated and reused.

Complementary: "Complementary" refers to two nucleotide sequences which comprise antiparallel nucleotide sequences capable of pairing with one another upon formation of hydrogen bonds between the complementary base residues in the antiparallel nucleotide sequences.

DNA shuffling: DNA shuffling is a method to rapidly, easily and efficiently introduce mutations or rearrangements, preferably randomly, in a DNA molecule or to generate exchanges of DNA sequences between two or more DNA molecules, preferably randomly. The DNA molecule resulting from DNA shuffling is a shuffled DNA molecule that is a non-naturally occurring DNA molecule derived from at least one template DNA molecule. The shuffled DNA encodes an enzyme modified with respect to the enzyme encoded by the template DNA, and preferably has an altered biological activity with respect to the enzyme encoded by the template DNA.

Enzyme activity: means herein the ability of an enzyme to catalyze the conversion of a substrate into a product. A substrate for the enzyme comprises the natural substrate of the enzyme but also comprises analogues of the natural substrate, which can also be converted, by the enzyme into a product or into an analogue of a product. The activity of the enzyme is measured for example by determining the amount of product in the reaction after a certain period of time, or by determining the amount of substrate remaining in the reaction mixture after a certain period of time. The activity of the enzyme is also measured by determining

the amount of an unused co-factor of the reaction remaining in the reaction mixture after a certain period of time or by determining the amount of used co-factor in the reaction mixture after a certain period of time. The activity of the enzyme is also measured by determining the amount of a donor of free energy or energy-rich molecule (e.g. ATP, phosphoenolpyruvate, acetyl phosphate or phosphocreatine) remaining in the reaction mixture after a certain period of time or by determining the amount of a used donor of free energy or energy-rich molecule (e.g. ADP, pyruvate, acetate or creatine) in the reaction mixture after a certain period of time.

Essential: An "essential" gene is a gene encoding a protein such as e.g. a biosynthetic enzyme, receptor, signal transduction protein, structural gene product, or transport protein that is essential to the growth or survival of the plant.

Expression cassette: "Expression cassette" as used herein means a DNA sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest but may also code for a functional RNA of interest, for example antisense RNA or a nontranslated RNA, in the sense or antisense direction. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular DNA sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, such as a plant, the promoter can also be specific to a particular tissue or organ or stage of development. In the case of a plastid expression cassette, for expression of the nucleotide sequence from a plastid genome, additional elements, i.e. ribosome binding sites, may be required.

Herbicide: a chemical substance used to kill or suppress the growth of plants, plant cells, plant seeds, or plant tissues.

Heterologous DNA Sequence: a DNA sequence not naturally associated with a host cell into which it is introduced, including non-naturally occurring multiple copies of a naturally occurring DNA sequence.

Homologous DNA Sequence: a DNA sequence naturally associated with a host cell.

Inhibitor: a chemical substance that inactivates the enzymatic activity of ENR-A, CBL, UROD, PBGD, or CPPO. The term "herbicide" is used herein to define an inhibitor when applied to plants, plant cells, plant seeds, or plant tissues.

Isogenic: plants which are genetically identical, except that they may differ by the presence or absence of a heterologous DNA sequence.

Isolated: in the context of the present invention, an isolated DNA molecule or an isolated enzyme is a DNA molecule or enzyme which, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated DNA molecule or enzyme may exist in a purified form or may exist in a non-native environment such as, for example, in a transgenic host cell.

Mature protein: protein which is normally targeted to a cellular organelle, such as a chloroplast, and from which the transit peptide has been removed.

Minimal Promoter: promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation. In the presence of a suitable transcription factor, the minimal promoter functions to permit transcription.

Modified Enzyme Activity: enzyme activity different from that which naturally occurs in a plant (i.e. enzyme activity that occurs naturally in the absence of direct or indirect manipulation of such activity by man), which is tolerant to inhibitors that inhibit the naturally occurring enzyme activity.

Native: A "native" refers to a gene which is present in the genome of the untransformed plant cell.

Plant: A "plant" refers to any plant or part of a plant at any stage of development. Therein are also included cuttings, cell or tissue cultures and seeds. As used in conjunction with the present invention, the term "plant tissue" includes, but is not limited to, whole plants, plant cells, plant organs, plant seeds, protoplasts, callus, cell cultures, and any groups of plant cells organized into structural and/or functional units.

Significant Increase: an increase in enzymatic activity that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 2-fold or greater of the activity of the wild-type enzyme in the presence of the inhibitor, more preferably an

increase by about 5-fold or greater, and most preferably an increase by about 10-fold or greater.

With respect to CBL, in its broadest sense, the term "substantially similar", when used herein with respect to a nucleotide sequence, means a nucleotide sequence corresponding to a reference nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence, e.g. where only changes in amino acids not affecting the polypeptide function occur. Desirably the substantially similar nucleotide sequence encodes the polypeptide encoded by the reference nucleotide sequence. The term "substantially similar" is specifically intended to include nucleotide sequences wherein the sequence has been modified to optimize expression in particular cells. The percentage of identity between the substantially similar nucleotide sequence and the reference nucleotide sequence desirably is at least 65%, more desirably at least 75%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, yet still more preferably at least 99%. Sequence comparisons are carried out using a Smith-Waterman sequence alignment algorithm (see e.g. Waterman, M.S. *Introduction to Computational Biology: Maps, sequences and genomes*. Chapman & Hall. London: 1995. ISBN 0-412-99391-0, or at <http://www-hho.usc.edu/software/segain/index.html>). The localS program, version 1.16, is used with following parameters: match: 1, mismatch penalty: 0.33, open-gap penalty: 2, extended-gap penalty: 2. A nucleotide sequence "substantially similar" to reference nucleotide sequence hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C. As used herein the term "CBL gene" refers to a DNA molecule comprising SEQ ID NO:3 or comprising a nucleotide sequence substantially similar to SEQ ID NO:3. Homologs of the CBL gene include nucleotide sequences that encode an amino acid sequence that is at least 30% identical to SEQ ID NO:4 as measured, using the parameters described below, wherein the amino acid sequence encoded by the homolog has the biological activity of the CBL protein.

With respect to CBL, the term "substantially similar", when used herein with respect to a protein, means a protein corresponding to a reference protein, wherein the protein has substantially the same structure and function as the reference protein, e.g. where only changes in amino acids sequence not affecting the polypeptide function occur. When used for a protein or an amino acid sequence the percentage of identity between the substantially similar and the reference protein or amino acid sequence desirably is at least 65%, more desirably at least 75%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, yet still more preferably at least 99%, using default BLAST analysis parameters. As used herein the term "CBL protein" refers to an amino acid sequence encoded by a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:3. Homologs of the CBL protein are amino acid sequences that are at least 30% identical to SEQ ID NO:4, as measured using the parameters described above, wherein the amino acid sequence encoded by the homolog has the biological activity of the CBL protein. With respect to UROD, in its broadest sense, the term "substantially similar", when used herein with respect to a nucleotide sequence, means a nucleotide sequence corresponding to a reference nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence. Desirably the substantially similar nucleotide sequence encodes the polypeptide encoded by the reference nucleotide sequence. The term "substantially similar" is specifically intended to include nucleotide sequences wherein the sequence has been modified to optimize expression in particular cells. Preferably, "substantially similar" refers to nucleotide sequences that encode a protein having at least 85% identity to SEQ ID NO:6, wherein said protein sequence comparisons are conducted using GAP analysis as described below. A nucleotide sequence "substantially similar" to the reference nucleotide sequence hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C. As used herein the term "UROD gene" refers to a DNA molecule comprising SEQ ID NO:5 or comprising a nucleotide sequence substantially similar to SEQ ID NO:5. Homologs

of the UROD gene include nucleotide sequences that encode an amino acid sequence that is at least 30% identical to SEQ ID NO:6 as measured, using the parameters described below, wherein the amino acid sequence encoded by the homolog has the biological activity of the UROD protein. Preferable are dicot homologs.

With respect to UROD, the term "substantially similar", when used herein with respect to a protein, means a protein corresponding to a reference protein, wherein the protein has substantially the same structure and function as the reference protein, e.g. where only changes in amino acids sequence not affecting the polypeptide function occur. When used for a protein or an amino acid sequence the percentage of identity between the substantially similar and the reference protein or amino acid sequence desirably is preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, yet still more preferably at least 99%, using default GAP analysis parameters with the University of Wisconsin GCG, SEQWEB application of GAP, based on the algorithm of Needleman and Wunsch (Needleman and Wunsch (1970) J Mol. Biol. 48: 443-453). As used herein the term "UROD protein" refers to an amino acid sequence encoded by a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:5. Homologs of the UROD protein are amino acid sequences that are at least 30% identical to SEQ ID NO:6, as measured using the parameters described above, wherein the amino acid sequence encoded by the homolog has the biological activity of the UROD protein. Preferable are dicot homologs.

With respect to PBGD, in its broadest sense, the term "substantially similar", when used herein with respect to a nucleotide sequence, means a nucleotide sequence corresponding to a reference nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence. Desirably the substantially similar nucleotide sequence encodes the polypeptide encoded by the reference nucleotide sequence. The term "substantially similar" is specifically intended to include nucleotide sequences wherein the sequence has been modified to optimize expression in particular cells. Preferably, "substantially similar" refers to nucleotide sequences that encode a protein having at least 85% identity to SEQ ID NO:8, wherein said protein sequence comparisons are conducted using GAP analysis as described below. A nucleotide sequence "substantially similar" to the reference nucleotide sequence hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium

dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C. As used herein the term "PBGD gene" refers to a DNA molecule comprising SEQ ID NO:7 or comprising a nucleotide sequence substantially similar to SEQ ID NO:7. Homologs of the PBGD gene include nucleotide sequences that encode an amino acid sequence that is at least 30% identical to SEQ ID NO:8 as measured, using the parameters described below, wherein the amino acid sequence encoded by the homolog has the biological activity of the PBGD protein. Preferable are dicot homologs.

With respect to PBGD, the term "substantially similar", when used herein with respect to a protein, means a protein corresponding to a reference protein, wherein the protein has substantially the same structure and function as the reference protein, e.g. where only changes in amino acids sequence not affecting the polypeptide function occur. When used for a protein or an amino acid sequence the percentage of identity between the substantially similar and the reference protein or amino acid sequence desirably is preferably at least 85%, more preferably at least 90%, still more preferably at least 95%, yet still more preferably at least 99%, using default GAP analysis parameters with the University of Wisconsin GCG, SEQWEB application of GAP, based on the algorithm of Needleman and Wunsch (Needleman and Wunsch (1970) J Mol. Biol. 48: 443-453). As used herein the term "PBGD protein" refers to an amino acid sequence encoded by a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:7. Homologs of the PBGD protein are amino acid sequences that are at least 30% identical to SEQ ID NO:8, as measured using the parameters described above, wherein the amino acid sequence encoded by the homolog has the biological activity of the PBGD protein. Preferable are dicot homologs.

With respect to CPPO, in its broadest sense, the term "substantially similar", when used herein with respect to a nucleotide sequence, means a nucleotide sequence corresponding to a reference nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence. Desirably, the substantially similar nucleotide sequence encodes the polypeptide encoded by the reference nucleotide sequence. The term "substantially similar" is specifically intended to include nucleotide sequences wherein the sequence has been modified to optimize expression in particular cells. Preferably, "substantially similar" refers to nucleotide sequences that encode a protein having at least

81% identity, more preferably at least 85% identity, still more preferably at least 90% identity, still more preferably at least 95% identity, yet still more preferably at least 99% identity, to SEQ ID NO:10, wherein said protein sequence comparisons are conducted using GAP analysis as described below. Also, "substantially similar" preferably also refers to nucleotide sequences having at least 75% identity, more preferably at least 80% identity, still more preferably at least 85% identity, still more preferably at least 90% identity, still more preferably 95% identity, yet still more preferably at least 99% identity, to SEQ ID NO:9, wherein said nucleotide sequence comparisons are conducted using GAP analysis as described below. A nucleotide sequence "substantially similar" to the reference nucleotide sequence preferably hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C. As used herein the term "CPPO gene" refers to a DNA molecule comprising SEQ ID NO:9 or comprising a nucleotide sequence substantially similar to SEQ ID NO:9. Homologs of the CPPO gene include nucleotide sequences that encode an amino acid sequence that is at least 50% identical to SEQ ID NO:10, more preferably at least 60% identical, still more preferably at least 65% identical, still more preferably at least 70%, yet still more preferably at least 80%, as measured, using the parameters described below, wherein the amino acid sequence encoded by the homolog has the biological activity of the CPPO protein.

With respect to CPPO, the term "substantially similar", when used herein with respect to a protein, means a protein corresponding to a reference protein, wherein the protein has substantially the same structure and function as the reference protein, e.g. where only changes in amino acids sequence not affecting the polypeptide function occur. When used for a protein or an amino acid sequence the percentage of identity between the substantially similar and the reference protein or amino acid sequence desirably is preferably at least 81%, more preferably at least 85%, still more preferably at least 90%, more preferably at least 95%, still more preferably at least 99% using default GAP analysis parameters with the University of Wisconsin GCG (version 10), SEQWEB application of GAP, based on the algorithm of Needleman and Wunsch (Needleman and Wunsch (1970) J Mol. Biol. 48: 443-

453). As used herein the term "CPPO protein" refers to an amino acid sequence encoded by a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:9. Homologs of the CPPO protein are amino acid sequences that are at least 50% identical to SEQ ID NO:10, as measured using the parameters described above, wherein the amino acid sequence encoded by the homolog has the biological activity of the CPPO protein.

With respect to ENR-A, in its broadest sense, the term "substantially similar", when used herein with respect to a nucleotide sequence, means a nucleotide sequence corresponding to a reference nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence. Desirably, the substantially similar nucleotide sequence encodes the polypeptide encoded by the reference nucleotide sequence. The term "substantially similar" is specifically intended to include nucleotide sequences wherein the sequence has been modified to optimize expression in particular cells. Preferably, "substantially similar" refers to nucleotide sequences that encode a protein having at least 90% identity, more preferably at least 95% identity, yet still more preferably at least 99% identity, to SEQ ID NO:2, wherein said protein sequence comparisons are conducted using GAP analysis as described below. Also, "substantially similar" preferably also refers to nucleotide sequences having at least 85% identity, more preferably at least 90% identity, still more preferably 95% identity, yet still more preferably at least 99% identity, to SEQ ID NO:1, wherein said nucleotide sequence comparisons are conducted using GAP analysis as described below. A nucleotide sequence "substantially similar" to the reference nucleotide sequence preferably hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M NaPO₄, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C. As used herein the term "ENR-A gene" refers to a DNA molecule comprising SEQ ID NO:1 or comprising a nucleotide sequence substantially similar to SEQ ID NO:1. Homologs of the ENR-A gene include nucleotide sequences that encode an amino acid sequence that is at least 30% identical to SEQ ID NO:2, more preferably at least 70%, still more preferably at least 85%, yet still more preferably at least 90%, as measured, using the parameters

described below, wherein the amino acid sequence encoded by the homolog has the biological activity of the ENR-A protein.

With respect to ENR-A, the term "substantially similar", when used herein with respect to a protein, means a protein corresponding to a reference protein, wherein the protein has substantially the same structure and function as the reference protein, e.g. where only changes in amino acids sequence not affecting the polypeptide function occur. When used for a protein or an amino acid sequence the percentage of identity between the substantially similar and the reference protein or amino acid sequence desirably is preferably at least 90%, more preferably at least 95%, still more preferably at least 99% using default GAP analysis parameters with the University of Wisconsin GCG (version 10), SEQWEB application of GAP, based on the algorithm of Needleman and Wunsch (Needleman and Wunsch (1970) J Mol. Biol. 48: 443-453). As used herein the term "ENR-A protein" refers to an amino acid sequence encoded by a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:1. Homologs of the ENR-A protein are amino acid sequences that are at least 30% identical to SEQ ID NO:2, as measured using the parameters described above, wherein the amino acid sequence encoded by the homolog has the biological activity of the ENR-A protein.

Substrate: a substrate is the molecule that an enzyme naturally recognizes and converts to a product in the biochemical pathway in which the enzyme naturally carries out its function, or is a modified version of the molecule, which is also recognized by the enzyme and is converted by the enzyme to a product in an enzymatic reaction similar to the naturally-occurring reaction.

Target gene: A "target gene" is any gene in a plant cell. For example, a target gene is a gene of known function or is a gene whose function is unknown, but whose total or partial nucleotide sequence is known. Alternatively, the function of a target gene and its nucleotide sequence are both unknown. A target gene is a native gene of the plant cell or is a heterologous gene which had previously been introduced into the plant cell or a parent cell of said plant cell, for example by genetic transformation. A heterologous target gene is stably integrated in the genome of the plant cell or is present in the plant cell as an extrachromosomal molecule, e.g. as an autonomously replicating extrachromosomal molecule.

Tolerance: the ability to continue essentially normal growth or function (i.e. no more than 5% of herbicide tolerant plants show phytotoxicity) when exposed to an inhibitor or herbicide in an amount sufficient to suppress the normal growth or function of native, unmodified plants.

Transformation: a process for introducing heterologous DNA into a cell, tissue, or plant. Transformed cells, tissues, or plants are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof.

Transgenic: stably transformed with a recombinant DNA molecule that preferably comprises a suitable promoter operatively linked to a DNA sequence of interest.

I. Plant ENR-A, CBL, UROD, PBGD, or CPPO Genes, respectively

In the present invention, the following abbreviations are used for the above plant genes. ENR-A is the abbreviation for enoyl-acyl carrier protein reductase; CBL is the abbreviation for cystathionine beta lyase; UROD is the abbreviation for uroporphyrinogen decarboxylase; PBGD is the abbreviation for porphobilinogen deaminase; and CPPO is the abbreviation for coproporphyrinogen oxidase.

CBL (EC 4.4.1.8) is an enzyme catalyzing a biochemical reaction required for the biosynthesis of the amino acid methionine. The methionine biosynthetic pathway in plants is outlined in Figure 1 of Ravenel et al., (1998) *Proc. Natl. Acad. Sci. USA* 95:7805-7812, incorporated herein by reference. This enzyme catalyzes the conversion of cystathionine to homocysteine by cleaving cystathionine to produce homocysteine, pyruvate, and ammonia. The sequence of a cDNA for the *Arabidopsis* CBL gene has been identified (EMBL accession # L40511; Ravanel et al. (1995) *Plant Mol. Biol.* 29: 875-882). The CBL gene has been cloned from other organisms, including *E. coli* (SWISS PROT accession # P06721), *S. typhimurium* (PIR accession # JV0020), *S. cerevisiae* (SWISS PROT accession # P43623), *B. subtilis* (GenPept accession # Z99110 AL009126), *Emericella nidulans* (GenPept accession # U28383), and human (GenPept accession # S52784). Results from GAP analysis of the above sequences show the following identities relative to *Arabidopsis thaliana*: *E. coli* (28% identical); *S. cerevisiae* (28% identical); humans (41% identical); *B. subtilis* (46% identical), and *Emericella nidulans* (47% identical).

UROD (EC 4.1.1.37) is an enzyme catalyzing a biochemical reaction required for the biosynthesis of porphyrin and heme. The porphyrin biosynthetic pathway in plants is outlined in Figure 1 of Reinbothe & Reinbothe, *Plant Physiol.* 111:1-7 (1996), incorporated herein by reference. This enzyme catalyzes the conversion of uroporphyrinogen III to coproporphyrinogen III. Coproporphyrinogen III is synthesized by plants, microorganisms, and animals as a precursor for the production of porphyrin and heme. In most organisms, heme is required as a prosthetic group for many enzymes, e.g. cytochrome oxidase. In plants, the porphyrin pathway produces chlorophyll (reviewed in Suzuki et al., *Annu. Rev.*

Genet. 31:61-89 (1997) and Reinbothe & Reinbothe, *Plant Physiol.* 111:1-7 (1996). The UROD gene has been cloned from many organisms, including *E. coli* (SWISS PROT accession # P29680), *S. cerevisiae* (SWISS PROT accession # P32347), humans (SWISS PROT accession # P06132), maize (SWISS PROT accession # O81220), and tobacco (SWISS PROT accession # Q42967).

PBGD (also known as hydroxymethylbilane synthase or preuroporphyrinogen synthase) (EC 4.3.1.8) is an enzyme catalyzing a biochemical reaction required for the biosynthesis of porphyrin and heme. The porphyrin biosynthetic pathway in plants is outlined in Figure 1 of Reinbothe & Reinbothe, *Plant Physiol.* 111:1-7 (1996), incorporated herein by reference. This enzyme catalyzes the condensation of four molecules of porphobilinogen to form hydroxymethylbilane. Hydroxymethylbilane is synthesized by plants, microorganisms, and animals as a precursor for the production of porphyrin and heme. In most organisms, heme is required as a prosthetic group for many enzymes, e.g. cytochrome oxidase. In plants, the porphyrin pathway produces chlorophyll (reviewed in Suzuki et al., *Annu. Rev. Genet.* 31:61-89 (1997) and Reinbothe & Reinbothe, *Plant Physiol.* 111:1-7 (1996)). The PBGD gene has been cloned from many organisms, including *E. coli* (SWISS PROT accession # P06983), *S. cerevisiae* (SWISS PROT accession # P28789), humans (SWISS PROT accession # P08397), pea (SWISS PROT accession #Q43082), *Methanococcus jannaschii* (SWISS PROT accession #Q57989), and *Arabidopsis thaliana* (SWISS PROT accession # Q43316) (Lim et al., *Plant Mol. Biol.* 26:863-872 (1994)). Results from GAP analysis of the above sequences show the following identities relative to *Arabidopsis thaliana*: *E. coli* (45% identical); *S. cerevisiae* (35% identical); humans (37% identical); pea (78% identical), and *Methanococcus jannaschii* (41% identical).

CPPO (EC 1.3.3.3) is an enzyme catalyzing a biochemical reaction required for the biosynthesis of porphyrin and heme. The porphyrin biosynthetic pathway in plants is outlined in Figure 1 of Reinbothe & Reinbothe, *Plant Physiol.* 111:1-7 (1996), incorporated herein by reference. This enzyme catalyzes the conversion of coproporphyrinogen III to protoporphyrinogen IX. Protoporphyrinogen IX is synthesized by plants, microorganisms, and animals as a precursor for the production of porphyrin and heme. In most organisms, heme is required as a prosthetic group for many enzymes, e.g. cytochrome oxidase. In plants, the porphyrin pathway produces chlorophyll (reviewed in Suzuki et al., *Annu. Rev. Genet.* 31:61-89 (1997) and Reinbothe & Reinbothe, *Plant Physiol.* 111:1-7 (1996)). The CPPO gene has been cloned from many organisms, including *E. coli*, aerobic form (SWISS PROT accession #P36553), *S. cerevisiae* (SWISS PROT accession # P11353), humans

(SWISS PROT accession # P36551), barley (SWISS PROT accession #Q42480), tobacco (SWISS PROT accession # Q42946), and soybean (SWISS PROT accession #P35055). Results from GAP analysis of the above sequences show the following identities at the amino acid level relative to *Arabidopsis thaliana*: *E. coli*, aerobic form (48% identical), *S. cerevisiae* (52% identical), humans (53% identical), barley (75% identical), tobacco (79% identical), and soybean (80% identical), and the following identities at the nucleotide level relative to *Arabidopsis thaliana*: barley (65% identical), soybean (73% identical).

ENR-A, also known as NADH enoyl-ACP reductase, (EC 1.3.1.9) is an enzyme catalyzing a biochemical reaction required for the final reducing step in the fatty acid biosynthesis cycle. The fatty acid biosynthetic pathway in plants is outlined in Figure 6.6 of Dey & Harborne, *Plant Biochemistry*, Academic Press (1997) incorporated herein by reference. This enzyme catalyzes the reduction of enoyl-acyl-ACP derivatives of carbon chain length from 4 to 16, by reducing a *trans*-unsaturated double bond to produce a saturated acyl-ACP, which can be elongated in the next condensation reaction. In plants, fatty acids act as energy stores, membrane constituents, and play key roles in metabolic control via second messenger signaling. The ENR-A gene has been cloned from many organisms, including *E. coli* (SWISS PROT accession #P29132), Petunia (GenBank accession # CAA05879), rice (GenBank accession # CAA05816), *Arabidopsis thaliana* (GenBank accession # CAA74175), and rape (SWISS PROT accession #P80030). Results from GAP analysis of the above sequences show the following identities at the amino acid level relative to *Arabidopsis thaliana*: *E. coli* (34% identical), Petunia (71% identical), rice (73% identical), and rape (90% identical), and the following identities at the nucleotide level relative to *Arabidopsis thaliana*: rape (85% identical). The sequences controlling the expression of the *Arabidopsis thaliana* enr-A gene have been described (de Boer, G.-J. et. al. (1999) Plant Mol. Biol. 39:1197-1207). The corresponding *E. coli* gene, *FabI*, has been shown to be inhibited by triclosan, an antimicrobial biocide (Heath, R.J. et al. (1999) J. Biol. Chem. 274:11110-11114).

In one aspect, the present invention is directed to a DNA molecule comprising a nucleotide sequence isolated from a plant source that encodes ENR-A, CBL, UROD, PBGD, or CPPO. In particular, the present invention provides a DNA molecule isolated from *Arabidopsis thaliana* that encodes ENR-A, CBL, UROD, PBGD, or CPPO, and DNA molecules substantially similar thereto that encode enzymes having ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively. The DNA coding sequence for ENR-A, CBL, UROD, PBGD, or

CPPO, from *Arabidopsis thaliana* is provided in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, respectively. The DNA sequence of the genomic sequence of the UROD, PBGD, CPPO, or ENR-A gene, from *Arabidopsis thaliana* is set forth in SEQ ID NO:21, SEQ ID NO:26, SEQ ID NO:29, or SEQ ID NO:11, respectively.

Based on Applicants' disclosure of the present invention, ENR-A, CBL, UROD, PBGD, or CPPO homologs, i.e. DNA sequences encoding ENR-A, CBL, UROD, PBGD, or CPPO enzymes, respectively, are isolated from the genome of any desired plant.

Alternatively, ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, can be isolated from any plant according to well known techniques based on their sequence similarity to the *Arabidopsis thaliana* coding sequences (SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, respectively) taught by the present invention. In these techniques, all or part of a known ENR-A, CBL, UROD, PBGD, or CPPO gene's coding sequence, respectively, is used as a probe that selectively hybridizes to other ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, present in a population of cloned genomic DNA fragments or cDNA fragments (i.e. genomic or cDNA libraries) from a chosen source organism. Such techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, e.g., Sambrook *et al.*, "Molecular Cloning", eds., Cold Spring Harbor Laboratory Press. (1989)) and amplification by PCR using oligonucleotide primers corresponding to sequence domains conserved among known ENR-A, CBL, UROD, PBGD, or CPPO enzyme's amino acid sequences, respectively (see, e.g. Innis *et al.*, "PCR Protocols, a Guide to Methods and Applications", Academic Press (1990)). These methods are particularly well suited to the isolation of ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, from organisms closely related to the organism from which the probe sequence is derived. The application of these methods using the *Arabidopsis* coding sequences as probes is well suited for the isolation of ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, from any source organism, preferably other plant species, including monocotyledons and dicotyledons.

The isolated ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, taught by the present invention can be manipulated according to standard genetic engineering techniques to suit any desired purpose. For example, an entire plant ENR-A, CBL, UROD, PBGD, or CPPO gene sequence, or portions thereof may be used as a probe capable of specifically hybridizing to coding sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include, e.g. sequences that are unique among plant ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, and are at least 10

nucleotides in length, preferably at least 20 nucleotides in length, and most preferably at least 50 nucleotides in length. Such probes are used to amplify and analyze ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, respectively, from a chosen organism via PCR. This technique is useful to isolate additional plant ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, respectively, from a desired organism or as a diagnostic assay to determine the presence of ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, in an organism. This technique also is used to detect the presence of altered ENR-A, CBL, UROD, PBGD, or CPPO gene sequences, associated with a particular condition of interest such as herbicide tolerance, poor health, etc.

ENR-A, CBL, UROD, PBGD, or CPPO, specific hybridization probes also are used to map the location of these native genes in the genome of a chosen plant using standard techniques based on the selective hybridization of the probe to genomic sequences. These techniques include, but are not limited to, identification of DNA polymorphisms identified or contained within the probe sequence, and use of such polymorphisms to follow segregation of the gene relative to other markers of known map position in a mapping population derived from self fertilization of a hybrid of two polymorphic parental lines (see e.g. Helentjaris *et al.*, *Plant Mol. Biol.* 5: 109 (1985); Sommer *et al.* *Biotechniques* 12:82 (1992); D'Ovidio *et al.*, *Plant Mol. Biol.* 15: 169 (1990)). While any plant ENR-A, CBL, UROD, PBGD, or CPPO gene sequence, is contemplated to be useful as a probe for mapping ENR-A, CBL, UROD, PBGD, or CPPO genes, respectively, preferred probes are those gene sequences from plant species more closely related to the chosen plant species, and most preferred probes are those gene sequences from the chosen plant species. Mapping of ENR-A, CBL, UROD, PBGD, or CPPO genes, in this manner is contemplated to be particularly useful for breeding purposes. For instance, by knowing the genetic map position of a mutant ENR-A, CBL, UROD, PBGD, or CPPO gene, that confers herbicide resistance, flanking DNA markers are identified from a reference genetic map (see, e.g., Helentjaris, *Trends Genet.* 3: 217 (1987)). During introgression of the herbicide resistance trait into a new breeding line, these markers are used to monitor the extent of linked flanking chromosomal DNA still present in the recurrent parent after each round of back-crossing.

ENR-A, CBL, UROD, PBGD, or CPPO, specific hybridization probes also are used to quantify levels of ENR-A, CBL, UROD, PBGD, or CPPO gene mRNA, respectively, in a plant using standard techniques such as Northern blot analysis. This technique is useful as a diagnostic assay to detect altered levels of ENR-A, CBL, UROD, PBGD, or CPPO gene

expression, respectively, that are associated with particular conditions such as enhanced tolerance to herbicides that target ENR-A, CBL, UROD, PBGD, or CPPO genes.

II. Essentiality of ENR-A, CBL, UROD, PBGD, or CPPO Genes, in Plants Demonstrated by Antisense Inhibition

As shown in the Examples below, the essentiality of ENR-A, CBL, UROD, PBGD, or CPPO genes, for normal plant growth and development has been demonstrated by antisense inhibition of expression of the ENR-A, CBL, UROD, PBGD, or CPPO gene, respectively, in International patent application no. WO 99/27119 entitled "Method and Compositions Useful for the Activation of Silent Transgenes", incorporated herein by reference. In this system, a hybrid transcription factor gene is made that comprises a DNA-binding domain and an activation domain. In addition, an activatable DNA construct is made that comprises a synthetic promoter operatively linked to an activatable DNA sequence. The hybrid transcription factor gene and synthetic promoter are selected such that the DNA binding domain of the hybrid transcription factor is capable of binding specifically to the synthetic promoter, which then activates expression of the activatable DNA sequence. A first plant is transformed with the hybrid transcription factor gene, and a second plant is transformed with the activatable DNA construct. The first plant and second plants are crossed to produce a progeny plant containing both the sequence encoding the hybrid transcription factor and the synthetic promoter, wherein the activatable DNA sequence is expressed in the progeny plant. In the preferred embodiment, the activatable DNA sequence is an antisense sequence capable of inactivating expression of ENR-A, CBL, UROD, PBGD, or CPPO, respectively. Hence, the progeny plant will be unable to normally express the endogenous gene.

This antisense validation system is especially useful for allowing expression of traits that might otherwise be unrecoverable as constitutively driven transgenes. For instance, foreign genes with potentially lethal effect or antisense genes or dominant-negative mutations designed to abolish function of essential genes, while of great interest in basic studies of plant biology, present inherent experimental problems. Decreased transformation frequencies are often cited as evidence of lethality associated with a particular constitutively driven transgene, but negative results of this type are laden with alternative trivial explanations. The antisense validation system is described in greater detail below:

A. Hybrid Transcription Factor Gene

A hybrid transcription factor gene for use in the antisense validation system described herein comprises DNA sequences encoding (1) a DNA-binding domain and (2) an activation domain that interacts with components of transcriptional machinery assembling at a promoter.

B. Activatable DNA Construct

An activatable DNA construct for use in the antisense validation system described herein comprises (1) a synthetic promoter operatively linked to (2) an activatable DNA sequence. The synthetic promoter comprises at least one DNA binding site recognized by the DNA binding domain of the hybrid transcription factor, and a minimal promoter, preferably a TATA element derived from a promoter recognized by plant cells. More particularly the TATA element is derived from a promoter recognized by the plant cell type into which the synthetic promoter will be incorporated. Desirably, the DNA binding site is repeated multiple times in the synthetic promoter so that the minimal promoter may be more effectively activated, such that the activatable DNA sequence associated with the synthetic promoter is more effectively expressed.

The activatable DNA sequence encompasses a DNA sequence, in this case ENR-A, CBL, UROD, PBGD, or CPPO, for which stable introduction and expression in a plant cell is desired. The activatable DNA sequence is operatively linked to the synthetic promoter to form the activatable DNA construct. The activatable DNA sequence in the activatable DNA construct is not expressed, i.e. is silent, in transgenic lines, unless a hybrid transcription factor capable of binding to and activating the synthetic promoter, is also present. The activatable DNA construct subsequently is introduced into cells, tissues or plants to form stable transgenic lines expressing the activatable DNA sequence, as described more fully below.

C. Transgenic Plants Containing the Hybrid Transcription Factor Gene or the Activatable DNA Construct

The antisense validation system utilizes a first plant containing the hybrid transcription factor gene and a second plant containing the activatable DNA construct. The hybrid transcription factor genes and activatable DNA constructs described above are introduced into the plants by methods well known and routinely used in the art, including but not limited to crossing, *Agrobacterium*-mediated transformation, protoplast transformation, Ti plasmid vectors, direct DNA uptake such as microprojectile bombardment, liposome mediated uptake, micro-

injection, etc. Transformants are screened for the presence and functionality of the transgenes according to standard methods known to those skilled in the art.

D. Transgenic Plants Containing Both the Hybrid Transcription Factor Gene and the Activatable DNA Construct

F1 plants containing both the hybrid transcription factor gene and the activatable DNA construct are generated by crossing said first and second plants and selected for the presence of an appropriate marker. In contrast to plants containing the activatable DNA construct alone, the F1 plants generate high levels of activatable DNA sequence expression product. Expression of ENR-A, CBL, UROD, PBGD, or CPPO antisense molecules, respectively, in such plants results in death or abnormal growth or development, indicating that ENR-A, CBL, UROD, PBGD, or CPPO, respectively, is essential for normal plant growth and development.

III. Recombinant Production of Plants ENR-A, CBL, UROD, PBGD, or CPPO Enzymes, and Uses Thereof

For recombinant production of a plant ENR-A, CBL, UROD, PBGD, or CPPO enzyme, in a host organism, a ENR-A, CBL, UROD, PBGD, or CPPO coding sequence, respectively, preferably a plant coding sequence, is inserted into an expression cassette designed for the chosen host and introduced into the host where it is recombinantly produced. The choice of specific regulatory sequences such as promoter, signal sequence, 5' and 3' untranslated sequences, and enhancer appropriate for the chosen host is within the level of skill of the routineer in the art. The resultant molecule, containing the individual elements operably linked in proper reading frame, is inserted into a vector capable of being transformed into the host cell. Suitable expression vectors and methods for recombinant production of proteins are well known for host organisms such as *E. coli*, yeast, and insect cells (see, e.g., Luckow and Summers, *Bio/Technol.* 6: 47 (1988)). Specific examples include plasmids such as pBluescript (Stratagene, La Jolla, CA; USA), pFLAG (International Biotechnologies, Inc., New Haven, CT, USA), pTrcHis (Invitrogen, La Jolla, CA, USA), and baculovirus expression vectors, e.g., those derived from the genome of *Autographica californica* nuclear polyhedrosis virus (AcMNPV). A preferred baculovirus/insect system is pVI11392/Sf21 cells (Invitrogen, La Jolla, CA, USA).

Recombinantly produced ENR-A, CBL, UROD, PBGD, or CPPO enzymes, respectively, are isolated and purified using a variety of standard techniques. The actual techniques used

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varies depending upon the host organism used, whether the enzyme is designed for secretion, and other such factors. Such techniques are well known to the skilled artisan (see, e.g. chapter 16 of Ausubel, F. et al., "Current Protocols in Molecular Biology", published by John Wiley & Sons, Inc. (1994).

Recombinantly produced ENR-A, CBL, UROD, PBGD, or CPPO enzymes are useful for a variety of purposes. For example, they are used in *in vitro* assays to screen known herbicidal chemicals, whose target has not been identified, to determine if they inhibit ENR-A, CBL, UROD, PBGD, or CPPO enzymes, respectively. Such *in vitro* assays also are useful as screens to identify new chemicals that inhibit such enzymatic activity and that are therefore novel herbicide candidates. Alternatively, recombinantly produced ENR-A, CBL, UROD, PBGD, or CPPO enzymes, are used to further characterize their association with known inhibitors in order to rationally design new inhibitory herbicides as well as herbicide tolerant forms of the enzymes.

In Vitro Inhibitor Assays: Discovery of Small Molecule Ligand that Interacts with the Gene Product of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, and SEQ ID NO:9

Once a protein has been identified as a potential herbicide target, the next step is to develop an assay that allows screening large number of chemicals to determine which ones interact with the protein. Although it is straightforward to develop assays for proteins of known function, developing assays with proteins of unknown functions is more difficult. This difficulty can be overcome by using technologies that can detect interactions between a protein and a compound without knowing the biological function of the protein. A short description of three methods is presented, including fluorescence correlation spectroscopy, surface-enhanced laser desorption/ionization, and biacore technologies.

Fluorescence Correlation Spectroscopy (FCS) theory was developed in 1972 but it is only in recent years that the technology to perform FCS became available (Madge et al. (1972) Phys. Rev. Lett., 29: 705-708; Maiti et al. (1997) Proc. Natl. Acad. Sci. USA, 94: 11753-11757). FCS measures the average diffusion rate of a fluorescent molecule within a small sample volume. The sample size can be as low as 10^3 fluorescent molecules and the sample volume as low as the cytoplasm of a single bacterium. The diffusion rate is a function of the mass of the molecule and decreases as the mass increases. FCS can therefore be applied to protein-ligand interaction analysis by measuring the change in mass and therefore in diffusion rate of a molecule upon binding. In a typical experiment, the target

to be analyzed is expressed as a recombinant protein with a sequence tag, such as a poly-histidine sequence, inserted at the N or C-terminus. The expression takes place in *E. coli*, yeast or insect cells. The protein is purified by chromatography. For example, the poly-histidine tag can be used to bind the expressed protein to a metal chelate column such as Ni²⁺ chelated on iminodiacetic acid agarose. The protein is then labeled with a fluorescent tag such as carboxytetramethylrhodamine or BODIPY® (Molecular Probes, Eugene, OR, USA). The protein is then exposed in solution to the potential ligand, and its diffusion rate is determined by FCS using instrumentation available from Carl Zeiss, Inc. (Thornwood, NY, USA). Ligand binding is determined by changes in the diffusion rate of the protein.

Surface-Enhanced Laser Desorption/Ionization (SELDI) was invented by Hutchens and Yip during the late 1980's (Hutchens and Yip (1993) *Rapid Commun. Mass Spectrom.* 7: 576-580). When coupled to a time-of-flight mass spectrometer (TOF), SELDI provides a mean to rapidly analyze molecules retained on a chip. It can be applied to ligand-protein interaction analysis by covalently binding the target protein on the chip and analyze by MS the small molecules that bind to this protein (Worrall et al. (1998) *Anal. Biochem.* 70: 750-756). In a typical experiment, the target to be analyzed is expressed as described for FCS. The purified protein is then used in the assay without further preparation. It is bound to the SELDI chip either by utilizing the poly-histidine tag or by other interaction such as ion exchange or hydrophobic interaction. The chip thus prepared is then exposed to the potential ligand via, for example, a delivery system capable to pipet the ligands in a sequential manner (autosampler). The chip is then submitted to washes of increasing stringency, for example a series of washes with buffer solutions containing an increasing ionic strength. After each wash, the bound material is analyzed by submitting the chip to SELDI-TOF. Ligands that specifically bind the target will be identified by the stringency of the wash needed to elute them.

Biacore relies on changes in the refractive index at the surface layer upon binding of a ligand to a protein immobilized on the layer. In this system, a collection of small ligands is injected sequentially in a 2-5 microlitre cell with the immobilized protein. Binding is detected by surface plasmon resonance (SPR) by recording laser light refracting from the surface. In general, the refractive index change for a given change of mass concentration at the surface layer, is practically the same for all proteins and peptides, allowing a single method to be applicable for any protein (Liedberg et al. (1983) *Sensors Actuators* 4: 299-304; Malmquist (1993) *Nature*, 361: 186-187). In a typical experiment, the target to be analyzed is expressed as described for FCS. The purified protein is then used in the assay without

further preparation. It is bound to the Biacore chip either by utilizing the poly-histidine tag or by other interaction such as ion exchange or hydrophobic interaction. The chip thus prepared is then exposed to the potential ligand via the delivery system incorporated in the instruments sold by Biacore (Uppsala, Sweden) to pipet the ligands in a sequential manner (autosampler). The SPR signal on the chip is recorded and changes in the refractive index indicate an interaction between the immobilized target and the ligand. Analysis of the signal kinetics on rate and off rate allows the discrimination between non-specific and specific interaction.

Also, an assay for small molecule ligands that interact with a polypeptide is an inhibitor assay. For example, such an inhibitor assay useful for identifying inhibitors of essential plant genes, such as plant ENR-A, CBL, UROD, PBGD, or CPPO genes, comprises the steps of:

- a) reacting a plant ENR-A, CBL, UROD, PBGD, or CPPO enzyme, and a substrate thereof in the presence of a suspected inhibitor of the enzyme's function;
- b) comparing the rate of enzymatic activity in the presence of the suspected inhibitor to the rate of enzymatic activity under the same conditions in the absence of the suspected inhibitor; and
- c) determining whether the suspected inhibitor inhibits the ENR-A, CBL, UROD, PBGD, or CPPO enzyme, respectively.

For example, the inhibitory effect on plant ENR-A, CBL, UROD, PBGD, or CPPO, may be determined by a reduction or complete inhibition of ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, in the assay. Such a determination may be made by comparing, in the presence and absence of the candidate inhibitor, the amount of substrate used or intermediate or product made during the reaction.

IV. *In Vivo* Inhibitor Assay

In one embodiment, a suspected herbicide, for example identified by *in vitro* screening, is applied to plants at various concentrations. The suspected herbicide is preferably sprayed on the plants. After application of the suspected herbicide, its effect on the plants, for example death or suppression of growth is recorded.

In another embodiment, an *in vivo* screening assay for inhibitors of the ENR-A, CBL, UROD, PBGD, or CPPO activity, uses transgenic plants, plant tissue, plant seeds or plant cells capable of overexpressing a nucleotide sequence having ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, wherein the ENR-A, CBL, UROD, PBGD, or CPPO gene

product, is enzymatically active in the transgenic plants, plant tissue, plant seeds or plant cells. The nucleotide sequence is preferably derived from an eukaryote, such as a yeast, but is preferably derived from a plant. In a further preferred embodiment, the nucleotide sequence is identical or substantially similar to the nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, or encodes an enzyme having ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, whose amino acid sequence is identical or substantially similar to the amino acid sequence set forth in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, or SEQ ID NO:10. In another preferred embodiment, the nucleotide sequence is derived from a prokaryote.

A chemical is then applied to the transgenic plants, plant tissue, plant seeds or plant cells and to the isogenic non-transgenic plants, plant tissue, plant seeds or plant cells, and the growth or viability of the transgenic and non-transformed plants, plant tissue, plant seeds or plant cells are determined after application of the chemical and compared. Compounds capable of inhibiting the growth of the non-transgenic plants, but not affecting the growth of the transgenic plants are selected as specific inhibitors of ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively.

V. Herbicide Tolerant Plants

Development of tolerance can allow application of a herbicide to a crop where its use was previously precluded or limited (e.g. to pre-emergence use) due to sensitivity of the crop to the herbicide. For example, U.S. Patent No. 4,761,373 to Anderson *et al.* is directed to plants resistant to various imidazolinone or sulfonamide herbicides. The resistance is conferred by an altered acetohydroxyacid synthase (AHAS) enzyme. U.S. Patent No. 4,975,374 to Goodman *et al.* relates to plant cells and plants containing a gene encoding a mutant glutamine synthetase (GS) resistant to inhibition by herbicides that were known to inhibit GS, e.g. phosphinothricin and methionine sulfoximine. U.S. Patent No. 5,013,659 to Bedbrook *et al.* is directed to plants expressing a mutant acetolactate synthase that renders the plants resistant to inhibition by sulfonylurea herbicides. U.S. Patent No. 5,162,602 to Somers *et al.* discloses plants tolerant to inhibition by cyclohexanedione and aryloxyphenoxypropanoic acid herbicides. The tolerance is conferred by an altered acetyl coenzyme A carboxylase (ACCase).

The present invention is further directed to plants, plant tissue, plant seeds, and plant cells tolerant to herbicides that inhibit the naturally occurring ENR-A, CBL, UROD, PBGD, or CPPO, in these plants, wherein the tolerance is conferred by altered ENR-A, CBL, UROD,

PBGD, or CPPO ENR-A enzyme activity, respectively. Altered ENR-A, CBL, UROD, PBGD, or CPPO enzyme activity, is conferred upon a plant according to the invention by increasing expression of wild-type herbicide-sensitive ENR-A, CBL, UROD, PBGD, or CPPO enzyme, by providing additional wild-type ENR-A, CBL, UROD, PBGD, or CPPO genes, to the plant, by expressing modified herbicide-tolerant ENR-A, CBL, UROD, PBGD, or CPPO enzymes, in the plant, or by a combination of these techniques. Representative plants include any plants to which these herbicides are applied for their normally intended purpose. Preferred are agronomically important crops such as cotton, soybean, oilseed rape, sugar beet, maize, rice, wheat, barley, oats, rye, sorghum, millet, turf, forage, turf grasses, and the like.

A. Increased Expression of Wild-Type ENR-A, CBL, UROD, PBGD, or CPPO Enzymes

Achieving altered ENR-A, CBL, UROD, PBGD, or CPPO enzyme activity, through increased expression results in a level of a ENR-A, CBL, UROD, PBGD, or CPPO enzyme, respectively, in the plant cell at least sufficient to overcome growth inhibition caused by the herbicide. The level of expressed enzyme generally is at least two times, preferably at least five times, and more preferably at least ten times the natively expressed amount. Increased expression is conferred in a number of ways, e.g., providing multiple copies of a wild-type ENR-A, CBL, UROD, PBGD, or CPPO gene, respectively; multiple occurrences of the coding sequence within the gene (*i.e.* gene amplification) or a mutation in the non-coding, regulatory sequence of the endogenous gene in the plant cell. Plants having such altered gene activity are obtained by direct selection in plants by methods known in the art (see, e.g. U.S. Patent No. 5,162,602, and U.S. Patent No. 4,761,373, and references cited therein). These plants also may be obtained by genetic engineering techniques known in the art. Increased expression of a herbicide-sensitive ENR-A, CBL, UROD, PBGD, or CPPO gene, also is accomplished by stably transforming a plant cell with a recombinant or chimeric DNA molecule comprising a promoter capable of driving expression of an associated structural gene in a plant cell operatively linked to a homologous or heterologous structural gene encoding the ENR-A, CBL, UROD, PBGD, or CPPO enzyme.

B. Expression of Modified Herbicide-Tolerant ENR-A, CBL, UROD, PBGD, or CPPO Enzymes

According to this embodiment, plants, plant tissue, plant seeds, or plant cells are stably transformed with a recombinant DNA molecule comprising a suitable promoter functional in plants operatively linked to a coding sequence encoding a herbicide tolerant form of a ENR-

A, CBL, UROD, PBGD, or CPPO enzyme. A herbicide tolerant form of the enzyme has at least one amino acid substitution, addition or deletion that confers tolerance to an amount of a herbicide effective to inhibit the unmodified, naturally occurring form of the ENR-A, CBL, UROD, PBGD, or CPPO enzyme. The transgenic plants, plant tissue, plant seeds, or plant cells thus created are selected by conventional selection techniques, whereby herbicide tolerant lines are isolated, characterized, and developed. Below are described methods for obtaining genes that encode herbicide tolerant forms of ENR-A, CBL, UROD, PBGD, or CPPO enzymes.

One strategy involves direct or indirect mutagenesis procedures on microbes. For instance, a genetically manipulatable microbe such as *E. coli* or *S. cerevisiae* may be subjected to random mutagenesis *in vivo* with mutagens such as UV light or ethyl or methyl methane sulfonate. Mutagenesis procedures are described, for example, in Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA (1972); Davis *et al.*, *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA (1980); Sherman *et al.*, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA (1983); and U.S. Patent No. 4,975,374. The microbe selected for mutagenesis contains a normal, inhibitor-sensitive ENR-A, CBL, UROD, PBGD, or CPPO gene, and is dependent upon the activity conferred by this gene. The mutagenized cells are grown in the presence of the inhibitor at concentrations that inhibit the unmodified gene. Colonies of the mutagenized microbe that grow better than the unmutagenized microbe in the presence of the inhibitor (i.e. exhibit resistance to the inhibitor) are selected for further analysis. ENR-A, CBL, UROD, PBGD, or CPPO genes, from these colonies are isolated, either by cloning or by PCR amplification, and their sequences are elucidated. Sequences encoding altered gene products are then cloned back into the microbe to confirm their ability to confer inhibitor tolerance.

A method of obtaining mutant herbicide-tolerant alleles of a plant ENR-A, CBL, UROD, PBGD, or CPPO gene, involves direct selection in plants. For example, the effect of a mutagenized ENR-A, CBL, UROD, PBGD, or CPPO gene, on the growth inhibition of plants such as *Arabidopsis*, soybean, or maize is determined by plating seeds sterilized by art-recognized methods on plates on a simple minimal salts medium containing increasing concentrations of the inhibitor. Such concentrations are in the range of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 110, 300, 1000 and 3000 parts per million (ppm). The lowest dose at which significant growth inhibition can be reproducibly detected is used for subsequent experiments.

Mutagenesis of plant material is utilized to increase the frequency at which resistant alleles occur in the selected population. Mutagenized seed material is derived from a variety of sources, including chemical or physical mutagenesis of seeds, or chemical or physical mutagenesis of pollen (Neuffer, In *Maize for Biological Research* Sheridan, ed. Univ. Press, Grand Forks, ND., pp. 61-64 (1982)), which is then used to fertilize plants and the resulting M₁ mutant seeds collected. Typically for *Arabidopsis* M₂ seeds, which are progeny seeds of plants grown from seeds mutagenized with chemicals, such as ethyl methane sulfonate, or with physical agents, such as gamma rays or fast neutrons, are plated at densities of up to 10,000 seeds/plate (10 cm diameter) on minimal salts medium containing an appropriate concentration of inhibitor to select for tolerance. Seedlings that continue to grow and remain green 7-21 days after plating are transplanted to soil and grown to maturity and seed set. Progeny of these seeds are tested for tolerance to a ENR-A, CBL, UROD, PBGD, or CPPO inhibitor. If the tolerance trait is dominant, plants whose seed segregate 3:1 / resistant:sensitive are presumed to have been heterozygous for the resistance at the M₂ generation. Plants that give rise to all resistant seed are presumed to have been homozygous for the resistance at the M₂ generation. Such mutagenesis on intact seeds and screening of their M₂ progeny seed can also be carried out on other species, for instance soybean (see, e.g. U.S. Pat. No. 5,084,082). Alternatively, mutant seeds to be screened for herbicide tolerance are obtained as a result of fertilization with pollen mutagenized by chemical or physical means.

Confirmation that the genetic basis of the herbicide tolerance is a modified ENR-A, CBL, UROD, PBGD, or CPPO gene, is ascertained as exemplified below. First, alleles of the ENR-A, CBL, UROD, PBGD, or CPPO gene, from plants exhibiting resistance to the inhibitor are isolated using PCR with primers based either upon conserved regions in the *Arabidopsis* cDNA coding sequences shown in SEQ ID NO:1 or, more preferably, based upon the unaltered ENR-A, CBL, UROD, PBGD, or CPPO gene sequence, from the plant used to generate tolerant alleles. After sequencing the alleles to determine the presence of mutations in the coding sequence, the alleles are tested for their ability to confer tolerance to the inhibitor on plants into which the putative tolerance-conferring alleles have been transformed. These plants are *Arabidopsis* plants or any other plant whose growth is susceptible to the inhibitors. Second, the ENR-A, CBL, UROD, PBGD, or CPPO genes, are mapped relative to known restriction fragment length polymorphisms (RFLPs) (See, for example, Chang et al. *Proc. Natl. Acad. Sci. USA* 85: 6856-6860 (1988); Nam et al., *Plant Cell* 1: 699-705 (1989). The tolerance trait is independently mapped using the same

markers. When tolerance is due to a mutation in the ENR-A, CBL, UROD, PBGD, or CPPO gene, the tolerance trait maps to a position indistinguishable from the position of the ENR-A, CBL, UROD, PBGD, or CPPO gene.

Another method of obtaining herbicide-tolerant alleles of a ENR-A, CBL, UROD, PBGD, or CPPO gene, is by selection in plant cell cultures. Explants of plant tissue, e.g. embryos, leaf disks, etc. or actively growing callus or suspension cultures of a plant of interest are grown on medium in the presence of increasing concentrations of a ENR-A, CBL, UROD, PBGD, or CPPO, inhibitor. Varying degrees of growth are recorded in different cultures. In certain cultures, fast-growing variant colonies arise that continue to grow even in the presence of normally inhibitory concentrations of inhibitor. The frequency with which such faster-growing variants occur can be increased by treatment with a chemical or physical mutagen before exposing the tissues or cells to the inhibitor. Putative tolerance-conferring alleles of the ENR-A, CBL, UROD, PBGD, or CPPO gene, are isolated and tested as described in the foregoing paragraphs. Those alleles identified as conferring herbicide tolerance may then be engineered for optimal expression and transformed into the plant. Alternatively, plants can be regenerated from the tissue or cell cultures containing these alleles.

Still another method involves mutagenesis of wild-type, herbicide sensitive plant ENR-A, CBL, UROD, PBGD, or CPPO genes, in bacteria or yeast, followed by culturing the microbe on medium that contains inhibitory concentrations of the inhibitor and then selecting those colonies that grow in the presence of the inhibitor. More specifically, a plant cDNA, such as the *Arabidopsis* cDNA encoding ENR-A (SEQ ID NO:1), CBL (SEQ ID NO:3), UROD (SEQ ID NO:5), PBGD (SEQ ID NO:7), or CPPO (SEQ ID NO:9), is cloned into a microbe that otherwise lacks the selected gene's activity. The transformed microbe is then subjected to *in vivo* mutagenesis or to *in vitro* mutagenesis by any of several chemical or enzymatic methods known in the art, e.g. sodium bisulfite (Shortle *et al.*, *Methods Enzymol.* 100:457-468 (1983); methoxylamine (Kadonaga *et al.*, *Nucleic Acids Res.* 13:1733-1745 (1985); oligonucleotide-directed saturation mutagenesis (Hutchinson *et al.*, *Proc. Natl. Acad. Sci. USA*, 83:710-714 (1986); or various polymerase misincorporation strategies (see, e.g. Shortle *et al.*, *Proc. Natl. Acad. Sci. USA*, 79:1588-1592 (1982); Shiraishi *et al.*, *Gene* 64:313-319 (1988); and Leung *et al.*, *Technique* 1:11-15 (1989). Colonies that grow in the presence of normally inhibitory concentrations of inhibitor are picked and purified by repeated restreaking. Their plasmids are purified and tested for the ability to confer tolerance to the inhibitor by retransforming them into the microbe lacking ENR-A, CBL,

UROD, PBGD, or CPPO gene activity. The DNA sequences of cDNA inserts from plasmids that pass this test are then determined.

Herbicide resistant ENR-A, CBL, UROD, PBGD, or CPPO proteins, are also obtained using methods involving *in vitro* recombination, also called DNA shuffling. By DNA shuffling, mutations, preferably random mutations, are introduced into nucleotide sequences encoding ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively. DNA shuffling also leads to the recombination and rearrangement of sequences within a ENR-A, CBL, UROD, PBGD, or CPPO gene, or to recombination and exchange of sequences between two or more different of ENR-A, CBL, UROD, PBGD, or CPPO genes, respectively. These methods allow for the production of millions of mutated ENR-A, CBL, UROD, PBGD, or CPPO coding sequences. The mutated genes, or shuffled genes, are screened for desirable properties, e.g. improved tolerance to herbicides and for mutations that provide broad spectrum tolerance to the different classes of inhibitor chemistry. Such screens are well within the skills of a routineer in the art.

In a preferred embodiment, a mutagenized ENR-A, CBL, UROD, PBGD, or CPPO gene, is formed from at least one template ENR-A, CBL, UROD, PBGD, or CPPO gene, wherein the template ENR-A, CBL, UROD, PBGD, or CPPO gene, has been cleaved into double-stranded random fragments of a desired size, and comprising the steps of adding to the resultant population of double-stranded random fragments one or more single or double-stranded oligonucleotides, wherein said oligonucleotides comprise an area of identity and an area of heterology to the double-stranded random fragments; denaturing the resultant mixture of double-stranded random fragments and oligonucleotides into single-stranded fragments; incubating the resultant population of single-stranded fragments with a polymerase under conditions which result in the annealing of said single-stranded fragments at said areas of identity to form pairs of annealed fragments, said areas of identity being sufficient for one member of a pair to prime replication of the other, thereby forming a mutagenized double-stranded polynucleotide; and repeating the second and third steps for at least two further cycles, wherein the resultant mixture in the second step of a further cycle includes the mutagenized double-stranded polynucleotide from the third step of the previous cycle, and the further cycle forms a further mutagenized double-stranded polynucleotide, wherein the mutagenized polynucleotide is a mutated ENR-A, CBL, UROD, PBGD, or CPPO gene, having enhanced tolerance to a herbicide which inhibits naturally occurring ENR-A, CBL, UROD, PBGD, or CPPO activity. In a preferred embodiment, the concentration of a single species of double-stranded random fragment in the population of double-stranded

random fragments is less than 1% by weight of the total DNA. In a further preferred embodiment, the template double-stranded polynucleotide comprises at least about 100 species of polynucleotides. In another preferred embodiment, the size of the double-stranded random fragments is from about 5 bp to 5 kb. In a further preferred embodiment, the fourth step of the method comprises repeating the second and the third steps for at least 10 cycles. Such method is described e.g. in Stemmer et al. (1994) Nature 370: 389-391, in US Patent 5,605,793, US Patent 5,811,238 and in Crameri et al. (1998) Nature 391: 288-291, as well as in WO 97/20078, and these references are incorporated herein by reference. In another preferred embodiment, any combination of two or more different ENR-A, CBL, UROD, PBGD, or CPPO genes, are mutagenized *in vitro* by a staggered extension process (StEP), as described e.g. in Zhao et al. (1998) Nature Biotechnology 16: 258-261. The two or more ENR-A, CBL, UROD, PBGD, or CPPO genes, respectively, are used as template for PCR amplification with the extension cycles of the PCR reaction preferably carried out at a lower temperature than the optimal polymerization temperature of the polymerase. For example, when a thermostable polymerase with an optimal temperature of approximately 72°C is used, the temperature for the extension reaction is desirably below 72°C, more desirably below 65°C, preferably below 60°C, more preferably the temperature for the extension reaction is 55°C. Additionally, the duration of the extension reaction of the PCR cycles is desirably shorter than usually carried out in the art, more desirably it is less than 30 seconds, preferably it is less than 15 seconds, more preferably the duration of the extension reaction is 5 seconds. Only a short DNA fragment is polymerized in each extension reaction, allowing template switch of the extension products between the starting DNA molecules after each cycle of denaturation and annealing, thereby generating diversity among the extension products. The optimal number of cycles in the PCR reaction depends on the length of the ENR-A, CBL, UROD, PBGD, or CPPO genes, to be mutagenized but desirably over 40 cycles, more desirably over 60 cycles, preferably over 80 cycles are used. Optimal extension conditions and the optimal number of PCR cycles for every combination of ENR-A, CBL, UROD, PBGD, or CPPO genes, are determined as described in using procedures well-known in the art. The other parameters for the PCR reaction are essentially the same as commonly used in the art. The primers for the amplification reaction are preferably designed to anneal to DNA sequences located outside of the ENR-A, CBL, UROD, PBGD, or CPPO genes, e.g. to DNA sequences of a vector comprising the ENR-A, CBL, UROD, PBGD, or CPPO genes, whereby the different ENR-A, CBL, UROD, PBGD, or CPPO genes, used in the PCR reaction are preferably comprised in separate vectors. The primers desirably

anneal to sequences located less than 500 bp away from ENR-A, CBL, UROD, PBGD, or CPPO sequences, preferably less than 200 bp away from the ENR-A, CBL, UROD, PBGD, or CPPO sequences, more preferably less than 120 bp away from the ENR-A, CBL, UROD, PBGD, or CPPO sequences. Preferably, the ENR-A, CBL, UROD, PBGD, or CPPO sequences, are surrounded by restriction sites, which are included in the DNA sequence amplified during the PCR reaction, thereby facilitating the cloning of the amplified products into a suitable vector. In another preferred embodiment, fragments of ENR-A, CBL, UROD, PBGD, or CPPO genes, having cohesive ends are produced as described in WO 98/05765. The cohesive ends are produced by ligating a first oligonucleotide corresponding to a part of a ENR-A, CBL, UROD, PBGD, or CPPO gene, to a second oligonucleotide not present in the gene or corresponding to a part of the gene not adjoining to the part of the gene corresponding to the first oligonucleotide, wherein the second oligonucleotide contains at least one ribonucleotide. A double-stranded DNA is produced using the first oligonucleotide as template and the second oligonucleotide as primer. The ribonucleotide is cleaved and removed. The nucleotide(s) located 5' to the ribonucleotide is also removed, resulting in double-stranded fragments having cohesive ends. Such fragments are randomly reassembled by ligation to obtain novel combinations of gene sequences.

Any ENR-A, CBL, UROD, PBGD, or CPPO gene, or any combination of ENR-A, CBL, UROD, PBGD, or CPPO genes, or homologs thereof, is used for *in vitro* recombination in the context of the present invention, for example, a ENR-A, CBL, UROD, PBGD, or CPPO gene, derived from a plant, such as, e.g. *Arabidopsis thaliana*, e.g. a ENR-A gene set forth in SEQ ID NO:1, CBL gene set forth in SEQ ID NO:3, UROD gene set forth in SEQ ID NO:5, PBGD gene set forth in SEQ ID NO:7, or CPPO gene set forth in SEQ ID NO:9. Whole ENR-A, CBL, UROD, PBGD, or CPPO genes, or portions thereof are used in the context of the present invention. The library of mutated ENR-A, CBL, UROD, PBGD, or CPPO genes, obtained by the methods described above are cloned into appropriate expression vectors and the resulting vectors are transformed into an appropriate host, for example a plant cell, an algae like *Chlamydomonas*, a yeast or a bacteria. An appropriate host requires ENR-A, CBL, UROD, PBGD, or CPPO gene product activity, for growth. Host cells transformed with the vectors comprising the library of mutated ENR-A, CBL, UROD, PBGD, or CPPO genes, are cultured on medium that contains inhibitory concentrations of the inhibitor and those colonies that grow in the presence of the inhibitor are selected. Colonies that grow in the presence of normally inhibitory concentrations of inhibitor are picked and purified by

repeated restreaking. Their plasmids are purified and the DNA sequences of cDNA inserts from plasmids that pass this test are then determined.

An assay for identifying a modified ENR-A, CBL, UROD, PBGD, or CPPO gene, that is tolerant to an inhibitor may be performed in the same manner as the assay to identify inhibitors of the ENR-A, CBL, UROD, PBGD, or CPPO enzyme, respectively, (Inhibitor Assay, above) with the following modifications: First, a mutant ENR-A, CBL, UROD, PBGD, or CPPO enzyme, is substituted in one of the reaction mixtures for the wild-type ENR-A, CBL, UROD, PBGD, or CPPO enzyme, respectively, of the inhibitor assay. Second, an inhibitor of wild-type enzyme is present in both reaction mixtures. Third, mutated activity (activity in the presence of inhibitor and mutated enzyme) and unmutated activity (activity in the presence of inhibitor and wild-type enzyme) are compared to determine whether a significant increase in enzymatic activity is observed in the mutated activity when compared to the unmutated activity. Mutated activity is any measure of activity of the mutated enzyme while in the presence of a suitable substrate and the inhibitor. Unmutated activity is any measure of activity of the wild-type enzyme while in the presence of a suitable substrate and the inhibitor. A significant increase is defined as an increase in enzymatic activity that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 2-fold or greater of the activity of the wild-type enzyme in the presence of the inhibitor, more preferably an increase by about 5-fold or greater, most preferably an increase by about 10-fold or greater.

In addition to being used to create herbicide-tolerant plants, genes encoding herbicide tolerant ENR-A, CBL, UROD, PBGD, or CPPO enzymes, also are used as selectable markers in plant cell transformation methods. For example, plants, plant tissue, plant seeds, or plant cells transformed with a transgene are transformed with a gene encoding an altered ENR-A, CBL, UROD, PBGD, or CPPO enzyme, capable of being expressed by the plant. The transformed cells are transferred to medium containing an ENR-A, CBL, UROD, PBGD, or CPPO inhibitor, in an amount sufficient to inhibit the survivability of plant cells not expressing the modified gene, wherein only the transformed cells will survive. The method is applicable to any plant cell capable of being transformed with a modified ENR-A, CBL, UROD, PBGD, or CPPO enzyme-encoding gene, and can be used with any transgene of interest. Expression of the transgene and the inhibitor-tolerant CBL, UROD, PBGD, CPPO, or ENR-A gene, can be driven by the same promoter functional in plant cells, or by separate promoters.

VI. Plant Transformation Technology

A wild-type or herbicide-tolerant form of the ENR-A, CBL, UROD, PBGD, or CPPO gene, can be incorporated in plant or bacterial cells using conventional recombinant DNA technology. Generally, this involves inserting a DNA molecule encoding the ENR-A, CBL, UROD, PBGD, or CPPO enzyme, into an expression system to which the DNA molecule is heterologous (i.e., not normally present) using standard cloning procedures known in the art. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences in a host cell containing the vector. A large number of vector systems known in the art can be used, such as plasmids, bacteriophage viruses and other modified viruses. The components of the expression system optionally are modified to increase expression. For example, truncated sequences, nucleotide substitutions or other modifications optionally are employed. Expression systems known in the art are used to transform virtually any crop plant cell under suitable conditions. Transformed cells are regenerated into whole plants such that the chosen form of the ENR-A, CBL, UROD, PBGD, or CPPO gene, confers herbicide tolerance in the transgenic plants.

A. Requirements for Construction of Plant Expression Cassettes

Gene sequences intended for expression in transgenic plants are first operably linked to a suitable promoter expressible in plants. Such expression cassettes optionally comprise further sequences required or selected for the expression of the transgene. Such sequences include, but are not restricted to, transcription terminators, extraneous sequences to enhance expression such as introns, vital sequences, and sequences intended for the targeting of the gene product to specific organelles and cell compartments. These expression cassettes are easily transferred to the plant transformation vectors described *infra*. The following is a description of various components of typical expression cassettes.

1. Promoters

The selection of the promoter used determines the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and the selection will reflect the desired location of accumulation of the gene product. Alternatively, the selected promoter may drive expression of the gene under various inducing conditions. Promoters vary in their strength, i.e., ability to promote transcription. Depending upon the host cell

system utilized, any one of a number of suitable promoters known in the art can be used. For example, for constitutive expression, the CaMV 35S promoter, the rice actin promoter, or the ubiquitin promoter may be used. For regulatable expression, the chemically inducible PR-1 promoter from tobacco or *Arabidopsis* may be used (see, e.g., U.S. Patent No. 5,689,044).

2. Transcriptional Terminators

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators are those that are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline synthase terminator and the pea *rbcS* E9 terminator. These can be used in both monocotyledons and dicotyledons.

3. Sequences for the Enhancement or Regulation of Expression

Numerous sequences are known to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants. For example, various intron sequences such as introns of the maize *Adh1* gene have been shown to enhance expression, particularly in monocotyledonous cells. In addition, a number of non-translated leader sequences derived from viruses also are known to enhance expression, and these are particularly effective in dicotyledonous cells.

4. Coding Sequence Optimization

The coding sequence of the selected gene optionally is genetically engineered by altering the coding sequence for optimal expression in the crop species of interest. Methods for modifying coding sequences to achieve optimal expression in a particular crop species are well known (see, e.g. Perlak et al., *Proc. Natl. Acad. Sci. USA* 88: 3324 (1991); and Koziel et al., *Bio/technol.* 11: 194 (1993); Fennoy and Bailey-Serres. *Nucl. Acids Res.* 21: 5294-5300 (1993). Methods for modifying coding sequences by taking into account codon usage in plant genes and in higher plants, green algae, and cyanobacteria are well known (see table 4 in: Murray et al. *Nucl. Acids Res.* 17: 477-498 (1989); Campbell and Gowri *Plant Physiol.* 92: 1-11(1990).

5. Targeting of the Gene Product Within the Cell

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail. For example, the targeting of gene products to the chloroplast is controlled by a signal sequence found at the amino terminal end of various proteins which is cleaved during chloroplast import to yield the mature protein (e.g. Comai et al. J. Biol. Chem. 263: 15104-15109 (1988)). Other gene products are localized to other organelles such as the mitochondrion and the peroxisome (e.g. Unger et al. Plant Molec. Biol. 13: 411-418 (1989)). The cDNAs encoding these products are manipulated to effect the targeting of heterologous gene products to these organelles. In addition, sequences have been characterized which cause the targeting of gene products to other cell compartments. Amino terminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, Plant Cell 2: 769-783 (1990)). Additionally, amino terminal sequences in conjunction with carboxy terminal sequences are responsible for vacuolar targeting of gene products (Shinshi et al. Plant Molec. Biol. 14: 357-368 (1990)). By the fusion of the appropriate targeting sequences described above to transgene sequences of interest one skilled in the art is able to direct the transgene product to any organelle or cell compartment.

B. Construction of Plant Transformation Vectors

Numerous transformation vectors available for plant transformation are known to those of ordinary skill in the plant transformation arts, and the genes pertinent to this invention are used in conjunction with any such vectors. The selection of vector will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene, which confers resistance to kanamycin and related antibiotics (Vieira & Messing Gene 19: 259-268 (1982); Bevan et al., Nature 304:184-187 (1983)), the *bar* gene, which confers resistance to the herbicide phosphinothricin (White et al., Nucl. Acids Res 18: 1062 (1990), Spencer et al. Theor. Appl. Genet 79: 625-631 (1990)), the *hph* gene, which confers resistance to the antibiotic hygromycin (Blochlinger & Diggelmann, Mol Cell Biol 4: 2929-2931), and the *dhfr* gene, which confers resistance to methotrexate (Bourouis et al., EMBO J. 2(7): 1099-1104 (1983)), and the EPSPS gene, which confers resistance to glyphosate (U.S. Patent Nos. 4,940,935 and 5,188,642).

1. Vectors Suitable for *Agrobacterium* Transformation

Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)). Typical vectors suitable for *Agrobacterium* transformation include the binary vectors pCIB200 and pCIB2001, as well as the binary vector pCIB10 and hygromycin selection derivatives thereof. (See, for example, U.S. Patent No. 5,639,949).

2. Vectors Suitable for non-*Agrobacterium* Transformation

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques that do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake (e.g. PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed. Typical vectors suitable for non-*Agrobacterium* transformation include pCIB3064, pSOG19, and pSOG35. (See, for example, U.S. Patent No. 5,639,949).

C. Transformation Techniques

Once the coding sequence of interest has been cloned into an expression system, it is transformed into a plant cell. Methods for transformation and regeneration of plants are well known in the art. For example, Ti plasmid vectors have been utilized for the delivery of foreign DNA, as well as direct DNA uptake, liposomes, electroporation, micro-injection, and microparticles. In addition, bacteria from the genus *Agrobacterium* can be utilized to transform plant cells.

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques that do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation

techniques, particle bombardment into callus tissue, as well as *Agrobacterium*-mediated transformation.

D. Plastid Transformation

In another preferred embodiment, a nucleotide sequence encoding a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity, is directly transformed into the plastid genome. Plastid expression, in which genes are inserted by homologous recombination into the several thousand copies of the circular plastid genome present in each plant cell, takes advantage of the enormous copy number advantage over nuclear-expressed genes to permit expression levels that can readily exceed 10% of the total soluble plant protein. In a preferred embodiment, the nucleotide sequence is inserted into a plastid targeting vector and transformed into the plastid genome of a desired plant host. Plants homoplasmic for plastid genomes containing the nucleotide sequence are obtained, and are preferentially capable of high expression of the nucleotide sequence.

Plastid transformation technology is for example extensively described in U.S. Patent Nos. 5,451,513, 5,545,817, 5,545,818, and 5,877,462 in PCT application no. WO 95/16783 and WO 97/32977, and in McBride et al. (1994) Proc. Natl. Acad. Sci. USA 91, 7301-7305, all incorporated herein by reference in their entirety. The basic technique for plastid transformation involves introducing regions of cloned plastid DNA flanking a selectable marker together with the nucleotide sequence into a suitable target tissue, e.g., using biotics or protoplast transformation (e.g., calcium chloride or PEG mediated transformation). The 1 to 1.5 kb flanking regions, termed targeting sequences, facilitate homologous recombination with the plastid genome and thus allow the replacement or modification of specific regions of the plastome. Initially, point mutations in the chloroplast 16S rRNA and rps12 genes conferring resistance to spectinomycin and/or streptomycin are utilized as selectable markers for transformation (Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990) Proc. Natl. Acad. Sci. USA 87, 8526-8530; Staub, J. M., and Maliga, P. (1992) Plant Cell 4, 39-45). The presence of cloning sites between these markers allowed creation of a plastid targeting vector for introduction of foreign genes (Staub, J.M., and Maliga, P. (1993) EMBO J. 12, 601-606). Substantial increases in transformation frequency are obtained by replacement of the recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, the bacterial *aadA* gene encoding the spectinomycin-detoxifying enzyme aminoglycoside-3'-adenyltransferase (Svab, Z., and

Maliga, P. (1993) *Proc. Natl. Acad. Sci. USA* 90, 913-917). Other selectable markers useful for plastid transformation are known in the art and encompassed within the scope of the invention.

VII. Breeding

The wild-type or altered form of a ENR-A, CBL, UROD, PBGD, or CPPO gene, of the present invention is utilized to confer herbicide tolerance to a wide variety of plant cells, including those of gymnosperms, monocots, and dicots. Although the gene can be inserted into any plant cell falling within these broad classes, it is particularly useful in crop plant cells, such as rice, wheat, barley, rye, corn, potato, carrot, sweet potato, sugar beet, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum and sugarcane.

The high-level expression of a wild-type ENR-A, CBL, UROD, PBGD, or CPPO gene, and/or the expression of herbicide-tolerant forms of a ENR-A, CBL, UROD, PBGD, or CPPO gene, conferring herbicide tolerance in plants, in combination with other characteristics important for production and quality, is incorporated into plant lines through breeding approaches and techniques known in the art.

Where a herbicide tolerant ENR-A, CBL, UROD, PBGD, or CPPO gene allele, is obtained by direct selection in a crop plant or plant cell culture from which a crop plant can be regenerated, it is moved into commercial varieties using traditional breeding techniques to develop a herbicide tolerant crop without the need for genetically engineering the allele and transforming it into the plant.

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, et al., Molecular Cloning, eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and by T.J. Silhavy, M.L. Berman, and

L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY , USA(1984) and by Ausubel, F.M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

Example 1: Regulation of the Expression of the CBL Gene

The CBL gene encodes a protein that carries out a step in the methionine biosynthesis pathway. CBL catalyzes the conversion of cystathionine to homocysteine (reviewed in Ravanel et al. (1998) *Proc. Natl. Acad. Sci. USA* 95: 7805-7812). The sequence of a cDNA for the *Arabidopsis* CBL gene has been identified (Ravanel et al. (1995) *Plant Mol. Biol.* 29: 875-882). The effect of the regulation of its expression in plants is tested using constructs for sense RNA expression (sense construct), antisense RNA expression (antisense construct) and antisense and sense RNA expression (antisense/sense construct).

A. Antisense construct: binary BASTA vector pJG261 (Guyer et al, *Genetics* (1998), 149: 633-639) is used containing a fragment from the pJG304 Δ Xhol vector (see below) with an insertion of part of the CBL gene in an antisense orientation (nucleotides #13-1159, GenBank accession #L40511).

pJG304 Δ Xhol: Plasmid pJG304 (Guyer et al, *Genetics* (1998), 149: 633-639) is partially digested with *Asp*718 to isolate a full-length linear fragment. This fragment is ligated with a molar excess of the 22 base oligonucleotide JG-L (5' GTA CCT CGA GTC TAG ACT CGA G 3'; SEQ ID NO:32). Restriction analysis is used to identify a clone with this linker inserted 5' to the GAL4 DNA binding site, and this plasmid is designated pJG304 Δ Xhol.

pJG304/aCBL: Plasmid pJG304 Δ Xhol is digested with *Nco*I and *Sac*I to excise the GUS gene. The GUS gene from pJG304 Δ Xhol is replaced with a CBL PCR product also digested with *Nco*I and *Sac*I. This product is generated using primers DG354 (5' GAT CGA GCT CCA CGA GAA CTG TCT CCG 3'; SEQ ID NO:14) and DG357 (5' TCA GCC ATG GGA AGA CAA GTA CAT TGC 3'; SEQ ID NO:15) and the pFL61 *Arabidopsis* cDNA library (Minet et al. (1992) *Plant J.* 2: 417-422) as a template. Plasmid pJG304/aCBL is constructed from the pJG304 Δ Xhol-digested vector ligated to the CBL PCR product.

pJG261/aCBL: pJG304/aCBL is cut with *Xba*I to excise the cassette containing the GAL4 DNA binding site/35S minimal promoter/antisense CBL/CaMV terminator fusion. This cassette is ligated into *Xba*I-digested pJG261 (Guyer et al, *Genetics* (1998), 149: 633-639), producing pJG261/aCBL.

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B. Sense construct: same as antisense construct, except the CBL fragment is in the opposite orientation. This construct contains the ATG start codon and most of the CBL ORF and serves as a control for regulation of the expression of the CBL gene.

pJG304/sCBL: Plasmid pJG304 Δ Xhol is digested with Ncol and SacI to excise the GUS gene. The GUS gene from pJG304 Δ Xhol is replaced with a CBL PCR product also digested with Ncol and SacI. This product is generated using primers CBL1 (5' CTT GCC ATG GCA CGA GAA CTG TCT CCG 3'; SEQ ID NO:8) and CBL2 (5' CAT GGA GCT CGA AGA CAA GTA CAT TGC A 3'; SEQ ID NO:17) and the pFL61 *Arabidopsis* cDNA library as a template. Plasmid pJG304/sCBL is constructed from the pJG304 Δ Xhol-digested vector ligated to the CBL PCR product.

pJG261/sCBL: pJG304/sCBL is cut with Xhol to excise the cassette containing the GAL4 DNA binding site/35S minimal promoter/sense CBL/CaMV terminator fusion. This cassette is ligated into Xhol-digested pJG261 (Guyer et al, *Genetics* (1998), 149: 633-639), producing pJG261/sCBL.

C. Antisense/sense construct: A CBL gene fragment (#13-1159, GenBank accession # L40511) in the sense orientation is inserted into the SalI site of vector pJG304 Δ Xhol downstream of the antisense orientation version of the CBL gene. A linker of about 10 bp is present between the two copies of CBL.

pJG304/dsCBL: Plasmid pJG304/aCBL is digested with SacI. A CBL PCR product also digested with SacI is inserted so that the inserted CBL gene is in the sense orientation. This product is generated using CBL2 (5' CAT GGA GCT CGA AGA CAA GTA CAT TGC A 3'; SEQ ID NO:17) and CBL3 (5' CAT CGA GCT CCT CTG TTT AAA CCA CGA GAA CTG TCT CCG TCG C 3'; SEQ ID NO:18) and the pFL61 *Arabidopsis* cDNA library as a template. The plasmid construct with the desired orientation of the inserted DNA is identified by digestion with HindIII. Plasmid pJG304/dsCBL is constructed from the pJG304/aCBL-digested vector ligated to the CBL PCR product. SURE2 (Stratagene, LaJolla, CA, USA) is used as the bacterial host to stabilize the construct.

pJG261/dsCBL: pJG304/dsCBL is cut with XbaI to excise the cassette containing the GAL4 DNA binding site/35S minimal promoter/antisense CBL/sense CBL/CaMV terminator fusion. This cassette is ligated into SpeI-digested pJG261 (Guyer et al, *Genetics* (1998), 149: 633-639), producing pJG261/dsCBL. XL1-BLUE MRF' (Stratagene, LaJolla, CA, USA) is used as the bacterial host to partially stabilize the construct. Unrearranged DNA for this construct is isolated by agarose gel purification.

D. Production of GAL4 Binding Site/Minimal CaMV 35S/CBL Transgenic Plants

The three described pJG261/CBL constructs are electro-transformed (Bio-Rad Laboratories, Hercules, CA) into *Agrobacterium tumefaciens recA* strain AGL1 (Lazo et al. (1991) *BioTechnology* 9: 963-967), and *Arabidopsis* plants (Ecotype Columbia) are transformed by infiltration (Bechtold et al., (1993) *C. R. Acad. Sci. Paris*, 316: 1188-1193). Seeds from the infiltrated plants are selected on germination medium (Murashige-Skoog salts at 4.3 g/liter, Mes at 0.5 g/liter, 1% sucrose, thiamine at 10 µg/liter, pyridoxine at 5 µg/liter, nicotinic acid at 5 µg/liter, myo-inositol at 1 mg/liter, pH 5.8) containing Basta at 15 mg/liter.

E. Comparison of the Inhibition of CBL Using a GAL4/C1 Transactivator and a GAL4 Binding Site/Minimal 35S Promoter

Transgenic plants containing a GAL4 binding site/minimal CaMV 35S promoter/ CBL construct are transplanted to soil and grown to maturity in the greenhouse. The presence of a transgenic CBL fragment in each line is confirmed by PCR. To test for the antisense construct, primers ASV1 (5' TTT GGA GAG GAC AGA CCT GC 3'; SEQ ID NO:19) and CBL3 (5' CAT CGA GCT CCT CTG TTT AAA CCA CGA GAA CTG TCT CCG TCG C 3'; SEQ ID NO:18) are used to verify the presence of an approximately 1200 bp product. Six transgenic lines with the antisense construct are identified. To test for the sense construct, primers ASV2 (5' GGA TTT TGG TTT TAG GAA TTA GAA 3'; SEQ ID NO:20) and CBL3 (5' CAT CGA GCT CCT CTG TTT AAA CCA CGA GAA CTG TCT CCG TCG C 3'; SEQ ID NO:18) are used to verify the presence of an approximately 1200 bp product. Thirteen transgenic lines with the sense construct are identified. To test for the antisense/sense construct, primers ASV2 (5' GGA TTT TGG TTT TAG GAA TTA GAA 3'; SEQ ID NO:20) and CBL3 (5' CAT CGA GCT CCT CTG TTT AAA CCA CGA GAA CTG TCT CCG TCG C 3'; SEQ ID NO:18) are used to verify the presence of an approximately 1200 bp product. In addition, to test for the antisense/sense construct, primers ASV1 (5' TTT GGA GAG GAC AGA CCT GC 3'; SEQ ID NO:19) and CBL3 (5' CAT CGA GCT CCT CTG TTT AAA CCA CGA GAA CTG TCT CCG TCG C 3'; SEQ ID NO:18) are used to verify the presence of an approximately 1200 bp product. Eleven transgenic lines with the antisense/sense construct are identified.

Flowers borne on the primary transformants are crossed to pollen from the homozygous GAL4/C1 transactivator line pAT53-103 (Guyer et al, *Genetics* (1998) 149: 633-649). F1 seeds are plated on MS + 2% sucrose medium (Murashige-Skoog salts at 4.3 g/liter, Mes at

0.5 g/liter, 2% sucrose). None of the lines comprising the antisense construct show an abnormal phenotype for the F1 progeny on plates. Two of thirteen lines comprising the sense construct show a weak phenotype for approximately half of the F1 progeny on each plate. The other eleven of thirteen lines comprising the sense construct do not show an abnormal phenotype for the F1 progeny on plates. Ten of eleven lines comprising the antisense/sense construct show phenotypes ranging from weak to strong for approximately half of the F1 progeny on each plate. Plants with a strong phenotype do not survive and have an increase in purple coloration, lose green pigmentation, and fail to form leaves after fourteen days on the plates. Plants with weaker phenotypes have some purple coloration, are paler green than normal, and form smaller leaves after fourteen days on the plates. Thus, the inventors are the first to demonstrate that the CBL gene is essential for the growth of a plant. Previously, it has been shown that tobacco mutants lacking CBL activity were unable to grow without exogenously-supplied methionine (Negruiti et al. (1985) *Mol. Gen. Genet.* 199: 330-337), but the molecular nature of the mutation has not been shown.

Example 2: Isolation of a cDNA Encoding UROD from *Arabidopsis*

Primers UROD-N-Nde (5'-GGGTTCCATATGTCAATCCTCAAGTCTC-3'; SEQ ID NO:22) and UROD-C-Not (5'-TTGCGCGGCCGCTTAATATCTAATTCTTGAGC-3'; SEQ ID NO:23) are designed to the 5' and 3' ends of the predicted UROD ORF (Open Reading Frame) from BAC genomic sequence (GenBank accession # AC002336), and PCR is performed using DNA from the pFL61 *Arabidopsis* Landsberg cDNA library (Minet et al. (1992) *Plant J.* 2: 417-422) as the template. Another RT-PCR is also performed using RNA isolated from *Arabidopsis* Col-0 leaf tissue. The resulting PCR products are digested with *Nde*I and *Not*I and ligated to pET 32a vector DNA (Stratagene, LaJolla, CA, USA) treated with the same restriction enzymes and sequenced. The UROD sequences from Col-0 and Landsberg are the same. Both are identical with the predicted ORF. The prior indicated exon/intron boundaries are: 48272..48787, 48874..48999, 49107..49295, 49391..49501, 49603..49727, 50182..50299, in the current version of GenBank accession # AC002336 annotated as 36805..36922, 37377..37501, 37603..37713, 37809..37997, 38105..38230, 38317..38832. The cDNA sequence is the same as the sequence predicted in the GenBank annotation, thus validating for the first time the putative open reading frame annotation. The *Arabidopsis* cDNA sequence encoding the UROD ORF is set forth in SEQ ID NO:5 and the encoded amino acid sequence is set forth in SEQ ID NO:6.

Example 3: Construction of a Vector Containing a GAL4 Binding Site/Minimal 35S CaMV Promoter Fused to Antisense UROD

pJG304/UD: Plasmid pJG304 Δ Xhol (Guyer et al, *Genetics* (1998), 149: 633-639) is digested with *Nco*I and *Bgl*II to excise the GUS gene. The GUS gene from pJG304 Δ Xhol is replaced with a UROD PCR product digested with *Afl*III (compatible with *Nco*I) and *Bam*HI (compatible with *Bgl*II). This product is generated using primers UROD-F2 (5'-CCCGGATCCATGTCAATCCTTCAAGTC-3'; SEQ ID NO:24) and UROD-R2 (5'-CCCACATGTATCTAATTCTTGAGC-3'; SEQ ID NO:25) and the pFL61 cDNA library as a template. Plasmid pJG304/UD is constructed from the pJG304 Δ Xhol digested vector ligated to the UROD PCR product.

Example 4: Plant Transformation Vectors for UROD Antisense Expression from the GAL4 Binding Site/CaMV Minimal 35S Promoter

pJG261/UD: pJG304/UD is cut with *Xba*I to excise the cassette containing the GAL4 DNA binding site/35S minimal promoter/antisense UROD/CaMV terminator fusion. This cassette is ligated into *Xba*I-digested pJG261 (Guyer et al, *Genetics* (1998), 149:633-639), producing pJG261/UD.

Example 5: Production Of GAL4 Binding Site/Minimal CaMV 35S Antisense UROD Transgenic Plants

pJG261/UD is electro-transformed (Bio-Rad Laboratories, Hercules, CA) into *Agrobacterium tumefaciens* strain GV3101, and *Arabidopsis* plants (Ecotype Columbia) are transformed by infiltration (Bechtold, et al., (1993) *C. R. Acad. Sci. Paris*, 316: 1188-93). Seeds from the infiltrated plants are selected on germination medium (Murashige-Skoog salts at 4.3 g/liter, Mes at 0.5 g/liter, 1% sucrose, thiamine at 10 μ g/liter, pyridoxine at 5 μ g/liter, nicotinic acid at 5 μ g/liter, myo-inositol at 1 mg/liter, pH 5.8) containing Basta at 15 mg/liter.

Example 6: Antisense Inhibition of UROD Using a GAL4/C1 Transactivator and a GAL4 Binding Site/Minimal CaMV 35S Promoter

Fifteen transgenic plants containing the GAL4 binding site/minimal CaMV 35S promoter/antisense UROD construct are transplanted to soil and grown to maturity in the greenhouse. Flowers borne on the primary transformants are crossed to pollen from the homozygous GAL4/C1 transactivator line pAT53-103 (Guyer et al, *Genetics* (1998) 149:633-649). F1 seeds are plated on MS + 2% sucrose medium (Murashige-Skoog salts at 4.3

g/liter, Mes at 0.5 g/liter, 2% sucrose) 6 lines segregate about 50% seedlings with a bleached lethal phenotype on plates. Thus, the inventors are the first to demonstrate that the UROD gene is essential for the growth of a dicot. Previously, it has been shown that maize plants homozygous for a loss-of-function mutation in this gene are dead (Hu et al, *Plant Cell* (1998) 10:1095-1105). In addition, it has been shown that tobacco plants, expressing a transgenic antisense construct, with 45% residual UROD activity exhibit necrosis, but not lethality (Mock et al, *Plant Physiol.* (1997) 113:1101-1112).

Example 7: Construction of a Vector Containing a GAL4 Binding Site/Minimal 35S CaMV Promoter Fused to Antisense PBGD

pJG304/UD: Plasmid pJG304 Δ Xhol (Guyer et al, *Genetics* (1998), 149: 633-639) is digested with *Nco*I and *Bgl*II to excise the GUS gene. The GUS gene from pJG304 Δ Xhol is replaced with a PBGD PCR product digested with *Bsp*HI (compatible with *Nco*I) and *Bgl*II. This product is generated using primers PORD-F2 (5'-CCC AGA TCT CCA TGG ATA TTG CTT CGT C-3'; SEQ ID NO:27) and PORD-R2 (5'-CCC TCA TGA AGA TAG CAA TTC TTG CCC-3'; SEQ ID NO:28) and the pFL61 *Arabidopsis* cDNA library (Minet et al. (1992) *Plant J.* 2: 417-422) as a template. Plasmid pJG304/PD is constructed from the pJG304 Δ Xhol digested vector ligated to the PBGD PCR product.

Example 8: Plant Transformation Vectors for PBGD Antisense Expression from the GAL4 Binding Site/CaMV Minimal 35S Promoter

pJG261/PD: pJG304/PD is cut with *Xba*I to excise the cassette containing the GAL4 DNA binding site/35S minimal promoter/antisense PBGD/CaMV terminator fusion. This cassette is ligated into *Xba*I-digested pJG261 (Guyer et al, *Genetics* (1998), 149:633-639), producing pJG261/PD.

Example 9: Production of GAL4 Binding Site/Minimal CaMV 35S Antisense PBGD Transgenic Plants

pJG261/PD is electro-transformed (Bio-Rad Laboratories, Hercules, CA) into *Agrobacterium tumefaciens* strain GV3101, and *Arabidopsis* plants (Ecotype Columbia) are transformed by infiltration (Bechtold, et al., (1993) *C. R. Acad. Sci. Paris*, 316: 1188-93). Seeds from the infiltrated plants are selected on germination medium (Murashige-Skoog salts at 4.3 g/liter, MES at 0.5 g/liter, 1% sucrose, thiamine at 10 µg/liter, pyridoxine at 5 µg/liter, nicotinic acid at 5 µg/liter, myo-inositol at 1 mg/liter, pH 5.8) containing Basta at 15 mg/liter.

Example 10: Antisense Inhibition of PBGD Using a GAL4/C1 Transactivator and a GAL4 Binding Site/Minimal CaMV 35S Promoter

Eighteen transgenic plants containing the GAL4 binding site/minimal CaMV 35S promoter/antisense PBGD construct are transplanted to soil and grown to maturity in the greenhouse. Flowers borne on the primary transformants are crossed to pollen from the homozygous GAL4/C1 transactivator line pAT53-103 (Guyer et al, *Genetics* (1998) 149:633-649). F1 seeds are plated on MS + 2% sucrose medium (Murashige-Skoog salts at 4.3 g/liter, Mes at 0.5 g/liter, 2% sucrose) eight lines segregate about 50% seedlings with a bleached lethal phenotype on plates. Thus, the inventors are the first to demonstrate that the PBGD gene is essential for the growth of a plant.

Example 11: Isolation of a cDNA Encoding CPPO from *Arabidopsis*

Primer CR73 (5' TTG ACC CTT CCT TCT ATC CCC GAT TC 3': SEQ ID NO:30) is designed to anneal to the complementary strand at 733-758 nucleotides from the 5' end of the start codon of the predicted CPPO ORF from the BAC F21B7 genomic sequence (GenBank accession # AC002560), and primer CR75 (5' GTT GCC ATG CCT TGT GCT GCT CTG TA 3': SEQ ID NO:31) is designed to anneal to the coding strand from 958-933 nucleotides from the 5' end of the start codon of the predicted CPPO ORF from the BAC F21B7 genomic sequence (GenBank accession # AC002560). 3' RACE is performed using CR73 primer and 5' RACE is performed using CR75 primer with second strand cDNA from *Arabidopsis thaliana* Ecotype Columbia as the template (Marathon cDNA Amplification Kit User Manual, Clontech). The resulting PCR products are TA-ligated and cloned (Original TA Cloning Kit, Invitrogen), and sequenced.

There are two differences between the sequence of the present invention and the genomic sequence in the prior art. First, the genomic sequence contains GG at positions 67872-67873. However, the inventors are the first to provide experimental evidence that the correct sequence contains only one G at position 67872. In addition, the genomic DNA that contains the CPPO ORF was not annotated correctly in the prior art with respect to the number of exons and the exon boundaries, the inventors are the first to provide experimental documentation of the correct ORF for the CPPO gene. The prior art indicates these exon boundaries: 66178..66702, 66782..66857, 66946..67040, 67126..67209, 67391..67478, 67571..67695, 67801..67896. In the sequence of the present invention, base 66178 marks the first base of the cDNA's start codon and base 68050 (using the numbering of the

deposited BAC which as indicated above is off by one nucleotide) marks the first base of the cDNA's stop codon. The 3' end of the exon 7 is 67900 (using the numbering of the deposited BAC which as indicated above is off by one nucleotide), and the 5' end of the exon containing the stop codon (i.e., exon 8) is 67984 (using the numbering of the deposited BAC which as indicated above is off by one nucleotide). The exon boundaries for the cDNA disclosed herein are: 66178..66702, 66782..66857, 66946..67040, 67126..67209, 67391..67478, 67571..67695, 67801..67900, 67984..68301.

The *Arabidopsis* cDNA sequence encoding the CPPO ORF is set forth in SEQ ID NO:9 and the encoded amino acid sequence is set forth in SEQ ID NO:10.

Example 12: Construction of a Vector Containing a GAL4 Binding Site/Minimal 35S CaMV Promoter Fused to Antisense CPPO

pJG304 Δ Xhol: Plasmid pJG304 (Guyer et al, *Genetics* (1998), 149: 633-639) is partially digested with *Asp718* to isolate a full-length linear fragment. This fragment is ligated with a molar excess of the 22 base oligonucleotide JG-L (5' GTA CCT CGA GTC TAG ACT CGA G 3'; SEQ ID NO:32). Restriction analysis is used to identify a clone with this linker inserted 5' to the GAL4 DNA binding site, and this plasmid is designated pJG304 Δ Xhol.

pJG304/CO: Plasmid pJG304 Δ Xhol is digested with *Ncol* and *Bg/II* to excise the GUS gene. The GUS gene from pJG304 Δ Xhol is replaced with a CPPO PCR product also digested with *Ncol* and *Bg/II*. This product is generated using primers CPPGO-F2 (5' CCC AGA TCT ATG GCT TCT CAC TCG TCG 3'; SEQ ID NO:33) and CPPGO-R2 (5' CAT GCC ATG GTA TTC CCA TCT TGC TGA AA 3'; SEQ ID NO:34) and the pFL61 *Arabidopsis* cDNA library (Minet et al. (1992) *Plant J.* 2: 417-422) as a template. Plasmid pJG304/CO is constructed from the pJG304 digested vector ligated to the CPPO PCR product.

Example 13: Plant Transformation Vectors For CPPO Antisense Expression From The GAL4 Binding Site/CaMV Minimal 35S Promoter

pJG261/CO: pJG304/CO is cut with *Xhol* to excise the cassette containing the GAL4 DNA binding site/35S minimal promoter/antisense CPPO/CaMV terminator fusion. This cassette is ligated into *Xhol*-digested pJG261 (Guyer et al, *Genetics* (1998), 149:633-639), producing pJG261/CO.

Example 14: Production Of GAL4 Binding Site/Minimal CaMV 35S Antisense CPPO Transgenic Plants

pJG261/CO is electro-transformed (Bio-Rad Laboratories, Hercules, CA) into *Agrobacterium tumefaciens* strain GV3101, and *Arabidopsis* plants (Ecotype Columbia) are transformed by infiltration (Bechtold, et al., (1993) *C. R. Acad. Sci. Paris*, 316: 1188-93). Seeds from the infiltrated plants are selected on germination medium (Murashige-Skoog salts at 4.3 g/liter, Mes at 0.5 g/liter, 1% sucrose, thiamine at 10 µg/liter, pyridoxine at 5 µg/liter, nicotinic acid at 5 µg/liter, myo-inositol at 1 mg/liter, pH 5.8) containing Basta at 15 mg/liter.

Example 15: Antisense Inhibition of CPPO Using a GAL4/C1 Transactivator and a GAL4 Binding Site/Minimal CaMV 35S Promoter

Fifteen transgenic plants containing the GAL4 binding site/minimal CaMV 35S promoter/antisense CPPO construct are transplanted to soil and grown to maturity in the greenhouse. Flowers borne on the primary transformants are crossed to pollen from the homozygous GAL4/C1 transactivator line pAT53-103 (Guyer et al, *Genetics* (1998) 149:633-649). F1 seeds are plated on MS + 2% sucrose medium (Murashige-Skoog salts at 4.3 g/liter, Mes at 0.5 g/liter, 2% sucrose) 13 lines segregate about 50% seedlings with a bleached lethal phenotype on plates. Thus, the inventors are the first to demonstrate that the CPPO gene is essential for the growth of a plant. Previously, it has been shown that tobacco plants expressing a transgenic antisense construct for this gene with 30-40% residual CPPO activity are sick (Kruse et al. *EMBO J* (1995) 14: 3712-3720).

Example 16: Construction of a Vector Containing a GAL4 Binding Site/Minimal 35S CaMV Promoter Fused to Antisense enoyl-ACP reductase (ENR-A)

pJG304/ENR-A: Plasmid pJG304ΔXhol (Guyer et al, *Genetics* (1998), 149: 633-639) is digested with *Nco*I and *Bgl*II to excise the GUS gene. The GUS gene from pJG304ΔXhol is replaced with a ENR-A PCR product digested *Nco*I and *Bgl*II. This product is generated using primers ENR-A-F2 (5'-CCC AGA TCT AAT GGC GGC TAC AGC AGC TT-3'; SEQ ID NO:12) and ENR-A-R2 (5'-CAT GCC ATG GCT AAT TCT TGC TGT TAA GG-3'; SEQ ID NO:13) and the pFL61 *Arabidopsis* cDNA library (Minet et al. (1992) *Plant J.* 2: 417-422) as a template. Plasmid pJG304/ENR-A is constructed from the pJG304ΔXhol digested vector ligated to the ENR-A PCR product.

Example 17: Plant Transformation Vectors For enoyl-ACP reductase (ENR-A) Antisense Expression from the GAL4 Binding Site/CaMV Minimal 35S Promoter

pJG261/ENR-A: pJG304/ENR-A is cut with *Xba*I to excise the cassette containing the GAL4 DNA binding site/35S minimal promoter/antisense enoyl-ACP reductase/CaMV terminator fusion. This cassette is ligated into *Spe*I-digested pJG261 (Guyer et al, *Genetics* (1998), 149:633-639), producing pJG261/ENR-A.

Example 18: Production of GAL4 Binding Site/Minimal CaMV 35S Antisense ENR-A Transgenic Plants

pJG261/ENR-A is electro-transformed (Bio-Rad Laboratories, Hercules, CA) into *Agrobacterium tumefaciens* strain GV3101, and *Arabidopsis* plants (Ecotype Columbia) are transformed by infiltration (Bechtold, et al., (1993) *C. R. Acad. Sci. Paris*, 316: 1188-93). Seeds from the infiltrated plants are selected on germination medium (Murashige-Skoog salts at 4.3 g/liter, MES at 0.5 g/liter, 1% sucrose, thiamine at 10 µg/liter, pyridoxine at 5 µg/liter, nicotinic acid at 5 µg/liter, myo-inositol at 1 mg/liter, pH 5.8) containing Basta at 15 mg/liter.

Example 19: Antisense Inhibition of ENR-A Using a GAL4/C1 Transactivator and a GAL4 Binding Site/Minimal CaMV 35S Promoter

Sixteen transgenic plants containing the GAL4 binding site/minimal CaMV 35S promoter/antisense enoyl-ACP reductase construct are transplanted to soil and grown to maturity in the greenhouse. Flowers borne on the primary transformants are crossed to pollen from the homozygous GAL4/C1 transactivator line pAT53-103 (Guyer et al, *Genetics* (1998) 149:633-649). F1 seeds are plated on MS + 2% sucrose medium (Murashige-Skoog salts at 4.3 g/liter, Mes at 0.5 g/liter, 2% sucrose). Two lines segregate about 50% seedling with a bleached phenotype on plates. These affected seedlings die shortly after transplanting to soil. Thus, the inventors are the first to demonstrate that the ENR-A gene is essential for the growth of a plant.

Example 20a: Expression of Recombinant CBL Protein in *E. coli*

The coding region of the protein, corresponding to the cDNA clone SEQ ID NO:3, is subcloned into an appropriate expression vector, and transformed into *E. coli* using the manufacturer's conditions. Specific examples include plasmids such as pBluescript (Stratagene, La Jolla, CA; USA), pFLAG (International Biotechnologies, Inc., New Haven, CT, USA), and pTrcHis (Invitrogen, La Jolla, CA, USA). *E. coli* is cultured, and expression of

CBL activity is confirmed. Protein conferring CBL activity is isolated using standard techniques.

Example 20b: Expression of Recombinant UROD Protein in *E. coli*

The coding region of the protein, corresponding to the cDNA clone SEQ ID NO:5, is subcloned into an appropriate expression vector, and transformed into *E. coli* using the manufacturer's conditions. Specific examples include plasmids such as pBluescript (Stratagene, La Jolla, CA, USA), pFLAG (International Biotechnologies, Inc., New Haven, CT, USA), and pTrcHis (Invitrogen, La Jolla, CA, USA). *E. coli* is cultured, and expression of UROD activity is confirmed. Protein conferring UROD activity is isolated using standard techniques.

Example 20c: Expression of Recombinant PBGD Protein in *E. coli*

The coding region of the protein, corresponding to the cDNA clone SEQ ID NO:7, is subcloned into an appropriate expression vector, and transformed into *E. coli* using the manufacturer's conditions. Specific examples include plasmids such as pBluescript (Stratagene, La Jolla, CA, USA), pFLAG (International Biotechnologies, Inc., New Haven, CT, USA), and pTrcHis (Invitrogen, La Jolla, CA, USA). *E. coli* is cultured, and expression of PBGD activity is confirmed. Protein conferring PBGD activity is isolated using standard techniques.

Example 20d: Expression of Recombinant CPPO Protein in *E. coli*

The coding region of the protein, corresponding to the cDNA clone SEQ ID NO:9, is subcloned into an appropriate expression vector, and transformed into *E. coli* using the manufacturer's conditions. Specific examples include plasmids such as pBluescript (Stratagene, La Jolla, CA, USA), pFLAG (International Biotechnologies, Inc., New Haven, CT, USA), and pTrcHis (Invitrogen, La Jolla, CA, USA). *E. coli* is cultured, and expression of CPPO activity is confirmed. Protein conferring CPPO activity is isolated using standard techniques.

Example 20e: Expression of Recombinant ENR-A Protein In *E. coli*

The coding region of the protein, corresponding to the cDNA clone SEQ ID NO:1, is subcloned into an appropriate expression vector, and transformed into *E. coli* using the manufacturer's conditions. Specific examples include plasmids such as pBluescript

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(Stratagene, La Jolla, CA, USA), pFLAG (International Biotechnologies, Inc., New Haven, CT, USA), and pTrcHis (Invitrogen, La Jolla, CA, USA). *E. coli* is cultured, and expression of ENR-A activity is confirmed. Protein conferring ENR-A activity is isolated using standard techniques.

Example 21: *In vitro* Recombination of ENR-A Genes by DNA Shuffling

The nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, respectively, is amplified by PCR. The resulting DNA fragment is digested by DNaseI treatment essentially as described (Stemmer et al. (1994) *PNAS* 91: 10747-10751) and the PCR primers are removed from the reaction mixture. A PCR reaction is carried out without primers and is followed by a PCR reaction with the primers, both as described (Stemmer et al. (1994) *PNAS* 91: 10747-10751). The resulting DNA fragments are cloned into pTRC99a (Pharmacia, Cat no: 27-5007-01) for use in bacteria, and transformed into a bacterial strain deficient in ENR-A, CBL, UROD, PBGD, or CPPO activity , respectively, by electroporation using the Biorad Gene Pulser and the manufacturer's conditions. The transformed bacteria are grown on medium that contains inhibitory concentrations of an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, and those colonies that grow in the presence of the inhibitor are selected. Colonies that grow in the presence of normally inhibitory concentrations of inhibitor are picked and purified by repeated restreaking. Their plasmids are purified and the DNA sequences of cDNA inserts from plasmids that pass this test are then determined. Alternatively, the DNA fragments are cloned into expression vectors for transient or stable transformation into plant cells, which are screened for differential survival and/or growth in the presence of an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively. In a similar reaction, PCR-amplified DNA fragments comprising the *Arabidopsis* ENR-A, CBL, UROD, PBGD, or CPPO gene, respectively, encoding the protein and PCR-amplified DNA fragments derived from or comprising another ENR-A, CBL, UROD, PBGD, or CPPO gene, respectively, are recombined *in vitro* and resulting variants with improved tolerance to the inhibitor are recovered as described above.

Example 22a: *In vitro* Recombination of CBL Genes by Staggered Extension Process

The *Arabidopsis* CBL gene and another CBL gene, or homologs thereof, or fragments thereof, are each cloned into the polylinker of a pBluescript vector. A PCR reaction is carried out essentially as described (Zhao et al. (1998) *Nature Biotechnology* 16: 258-261)

using the "reverse primer" and the "M13 -20 primer" (Stratagene Catalog). Amplified PCR fragments are digested with appropriate restriction enzymes and cloned into pTRC99a and mutated CBL genes are screened as described in Example 21.

Example 22b: *In vitro* Recombination of UROD Genes by Staggered Extension Process

The *Arabidopsis* UROD gene and another UROD gene, or homologs thereof, or fragments thereof, are each cloned into the polylinker of a pBluescript vector. A PCR reaction is carried out essentially as described (Zhao et al. (1998) *Nature Biotechnology* 16: 258-261) using the "reverse primer" and the "M13 -20 primer" (Stratagene Catalog). Amplified PCR fragments are digested with appropriate restriction enzymes and cloned into pTRC99a and mutated UROD genes are screened as described in Example 21.

Example 22c: *In vitro* Recombination of PBGD Genes by Staggered Extension Process

The *Arabidopsis* PBGD gene and another PBGD gene, or homologs thereof, or fragments thereof, are each cloned into the polylinker of a pBluescript vector. A PCR reaction is carried out essentially as described (Zhao et al. (1998) *Nature Biotechnology* 16: 258-261) using the "reverse primer" and the "M13 -20 primer" (Stratagene Catalog). Amplified PCR fragments are digested with appropriate restriction enzymes and cloned into pTRC99a and mutated PBGD genes are screened as described in Example 21.

Example 22d: *In vitro* Recombination of CPPO Genes by Staggered Extension Process

The *Arabidopsis* CPPO gene and another CPPO gene, or homologs thereof, or fragments thereof, are each cloned into the polylinker of a pBluescript vector. A PCR reaction is carried out essentially as described (Zhao et al. (1998) *Nature Biotechnology* 16: 258-261) using the "reverse primer" and the "M13 -20 primer" (Stratagene Catalog). Amplified PCR fragments are digested with appropriate restriction enzymes and cloned into pTRC99a and mutated CPPO genes are screened as described in Example 21.

Example 22e: *In vitro* Recombination of ENR-A Genes by Staggered Extension Process

The *Arabidopsis* ENR-A gene and another ENR-A gene, or homologs thereof, or fragments thereof, are each cloned into the polylinker of a pBluescript vector. A PCR reaction is carried out essentially as described (Zhao et al. (1998) *Nature Biotechnology* 16: 258-261) using the "reverse primer" and the "M13 -20 primer" (Stratagene Catalog). Amplified PCR fragments are digested with appropriate restriction enzymes and cloned into pTRC99a and mutated ENR-A genes are screened as described in Example 21.

Example 23: *In Vitro* Binding Assays

Recombinant ENR-A, CBL, UROD, PBGD, or CPPO protein, respectively, is obtained, for example, according to Example 20. The protein is immobilized on chips appropriate for ligand binding assays using techniques which are well known in the art. The protein immobilized on the chip is exposed to sample compound in solution according to methods well known in the art. While the sample compound is in contact with the immobilized protein measurements capable of detecting protein-ligand interactions are conducted. Examples of such measurements are SELDI, biacore and FCS, described above. Compounds found to bind the protein are readily discovered in this fashion and are subjected to further characterization.

Various modifications of the invention described herein will become apparent to those skilled in the art. Such modifications are intended to fall within the scope of the appended claims.

Example 24: CBL Activity Assay

The CBL activity assay is derived from Stintjes et al. (1992) *Anal. Biochem.* 206, 334-343. The reaction volumes are preferably the ones described below, but can be varied depending on the experimental requirements. $0.01\text{-}1.0 \times 10^{-3}$ unit of an enzyme having CBL activity (one unit of activity is defined as the amount of enzyme required to produce 1 mmol/min of product) and 0.5-5 mM, but preferably 1 mM L(+)-cystathionine (cyn) are mixed in a final volume of 10 mL 10 mM Tris-HCl (pH 7.0-9.0, but preferably 8.5) and 1-20 mM, but preferably 10 mM pyridoxal 5'-phosphate. The production of pyruvate is determined preferably according to Stintjes et al. (1992) *Anal. Biochem.* 206, 334-343 by adding 5 mL of 20 mM o-phenylenediamine in 0.6 M hydrochloric acid. Fluorescence intensity is measured for the solution with an excitation wavelength of 410 ± 10 nm and an emission wavelength of 535 ± 10 nm. Alternatively, the absorbance of the solution may be measured with a wavelength of 410 ± 10 nm.

Alternatively, pyruvate formation is quantified by a coupled reaction procedure. In this case, 0.5 units of lactate dehydrogenase and 0.2 mM NADH are added and the fluorescence intensity of the solution is measured with an excitation wavelength of 340 ± 10 nm and an emission wavelength of 410 ± 10 nm. Alternatively, the absorbance of the solution may be measured at 340 nm. Other ways to measure the activity of this enzyme known in the art may be used.

Example 25: *In Vitro* Functional Assay for PBGD Activity

Recombinant PBGD protein is obtained, for example, according to Example 5. The protein can be used in a functional PBGD activity assay as described in Jones and Leadbeater (1997) *Meth. Enz.* 281, 327-336. The reaction volumes are preferably the ones described below, but can be varied depending on the experimental requirements. $0.01\text{-}1.0 \times 10^{-3}$ unit of an enzyme having PBGD activity (one unit of activity is defined as the amount of enzyme required to produce 1 mmol/min of product) and 0.01-5 mM, but preferably 0.05 mM, porphobilinogen are mixed in a final volume of 10 mL 20 mM Tris-HCl (pH 7.0-9.0, but preferably 8.0) and 0.1-10 mM, but preferably 2 mM, dithiothreitol. The production of hydroxymethylbilane is determined indirectly preferably according to Jones and Leadbeater (1997) *Meth. Enz.* 281, 327-336 by adding 5 mL of 5 mM hydrochloric acid followed by 5 mL of 0.1% benzoquinone in methanol. Fluorescence intensity is measured for the solution with an excitation wavelength of 405 ± 10 nm and an emission wavelength of 620 ± 10 nm.

Example 26: *In vitro* Enzymatic Assay for CPPO Activity

Recombinant CPPO protein is obtained, for example, according to Example 6. The protein is used for *in vitro* enzymatic assays. At least three procedures are used by one skilled in the art. First, CPPO is combined with a protoporphyrinogen oxidase. In this procedure, coproporphyrinogen III is converted to protoporphyrinogen IX by CPPO and protoporphyrinogen IX is converted to protoporphyrin IX by protoporphyrinogen oxidase (Labbe, Camadro, and Chambon (1985) *Anal. Biochem.*, 149: 248-260). The formation of protoporphyrin IX is measured colorimetrically or fluorimetrically. Alternatively, CPPO is assayed singularly by converting protoporphyrinogen IX, the product of the CPPO enzymatic activity, to protoporphyrin IX chemically using an oxidizing agent known to one skilled in the art (Yoshinga (1997) *Meth. Enz.* 281: 355-367). The formation of protoporphyrin IX can be measured colorimetrically or fluorimetrically. Additionally, the formation of

protoporphyrinogen IX from coproporphyrinogen III is measured by HPLC (Rossi, Garcia-Webb, and Costin (1989) Clin. Chim. Acta 181: 115-117).

Example 27: Plastid Transformation

Transformation vectors

For expression of a nucleotide sequence encoding a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity, respectively, encoding in plant plastids, plastid transformation vector pPH143 or pPH145 (WO 97/32011) is used; and this reference is incorporated herein by reference. The nucleotide sequence is inserted into pPH143 thereby replacing the PROTOX coding sequence. This vector is then used for plastid transformation and selection of transformants for spectinomycin resistance. Alternatively, the nucleotide sequence is inserted in pPH143 so that it replaces the *aadH* gene. In this case, transformants are selected for resistance to PROTOX inhibitors.

Plastid Transformation

Seeds of *Nicotiana tabacum* c.v. 'Xanthi nc' are germinated seven per plate in a 1" circular array on T agar medium and bombarded 12-14 days after sowing with 1 μm tungsten particles (M10, Biorad, Hercules, CA) coated with DNA from plasmids pPH143 and pPH145 essentially as described (Svab, Z. and Maliga, P. (1993) *Proc. Natl. Acad. Sci. USA* 90, 913-917). Bombarded seedlings are incubated on T medium for two days after which leaves are excised and placed abaxial side up in bright light (350-500 μmol photons/ m^2/s) on plates of RMOP medium (Svab, Z., Hajdukiewicz, P. and Maliga, P. (1990) *Proc. Natl. Acad. Sci. USA* 87, 8526-8530) containing 500 $\mu\text{g}/\text{ml}$ spectinomycin dihydrochloride (Sigma, St. Louis, MO). Resistant shoots appearing underneath the bleached leaves three to eight weeks after bombardment are subcloned onto the same selective medium, allowed to form callus, and secondary shoots isolated and subcloned. Complete segregation of transformed plastid genome copies (homoplasmicity) in independent subclones is assessed by standard techniques of Southern blotting (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor). Homoplasmic shoots are rooted aseptically on spectinomycin-containing MS/IBA medium (McBride, K. E. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 7301-7305) and transferred to the greenhouse. Various modifications of the invention described herein will become apparent to those skilled in the art. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. An isolated DNA molecule comprising a nucleotide sequence encoding an amino acid sequence substantially similar to SEQ ID NO:6 or SEQ ID NO:10.
2. The DNA molecule of claim 1, wherein said nucleotide sequence is substantially similar to SEQ ID NO:5 or SEQ ID NO:9.
3. The DNA molecule according to claim 1, wherein said nucleotide sequence is a plant nucleotide sequence.
4. The DNA molecule of claim 1, wherein the amino acid sequence has UROD or CPPO activity.
5. A polypeptide comprising an amino acid sequence encoded by a nucleotide sequence substantially similar to SEQ ID NO:5 or SEQ ID NO:9.
6. The polypeptide of claim 5, wherein said amino acid sequence is substantially similar to SEQ ID NO:6 or SEQ ID NO:10.
7. The polypeptide of claim 5, wherein said amino acid sequence has UROD or CPPO activity.
8. A polypeptide comprising an amino acid sequence comprising at least 20 consecutive amino acid residues of the amino acid sequence of SEQ ID NO:6 or SEQ ID NO:10.
9. An expression cassette comprising a promoter operatively linked to a DNA molecule according to claim 1.
10. A recombinant vector comprising an expression cassette according to claim 9.
11. A host cell comprising a DNA molecule according to claim 1.
12. A host cell according to claim 11, wherein said host cell is selected from the group consisting of an insect cell, a yeast cell, a prokaryotic cell and a plant cell.
13. A plant or seed comprising a plant cell according to claim 12.
14. A plant of claim 13, wherein said plant is tolerant to an inhibitor of UROD or CPPO activity.
15. An expression cassette comprising a promoter functional in a eukaryote operatively linked to a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:1 or SEQ ID NO:7.
16. A recombinant vector comprising an expression cassette according to claim 15.
17. A host cell comprising an expression cassette according to claim 15.
18. A host cell according to claim 17, wherein said host cell is selected from the group consisting of an insect cell, a yeast cell, a prokaryotic cell and a plant cell.

19. A plant cell comprising an isolated DNA molecule comprising a nucleotide sequence identical or substantially similar to SEQ ID NO:1 or SEQ ID NO:7.
20. A plant or seed comprising a plant cell of claim 19.
21. The plant of claim 20, wherein said plant is tolerant to an inhibitor of ENR-A or PBGD activity.
22. A host cell comprising an expression cassette, comprising a promoter operatively linked to an isolated DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:3, wherein said host cell is an eukaryotic cell.
23. A host cell according to claim 22, wherein said host cell is selected from the group consisting of an insect cell, a yeast cell, and a plant cell.
24. A plant or seed comprising a plant cell of claim 23.
25. A plant of claim 24, wherein said plant is tolerant to an inhibitor of CBL activity.
26. A method comprising:
 - a) combining a polypeptide comprising the amino acid sequence encoded by a nucleotide sequence substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, or a homolog thereof, and a compound to be tested for the ability to interact with said polypeptide, under conditions conducive to interaction; and
 - b) selecting a compound identified in step (a) that is capable of interacting with said polypeptide.
27. The method according to claim 26, further comprising:
 - c) applying a compound selected in step (b) to a plant to test for herbicidal activity; and
 - d) selecting compounds having herbicidal activity.
28. A compound identifiable by the method of claim 26.
29. A compound having herbicidal activity identifiable by the method of claim 27.
30. A process of identifying an inhibitor of ENR-A, CBL, UROD, PBGD, or CPPO activity comprising:
 - a) introducing a DNA molecule comprising a nucleotide sequence substantially similar to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:9, and encoding a polypeptide having ENR-A, CBL, UROD, PBGD, or CPPO activity, or a homolog thereof, into a plant cell, such that said sequence is functionally expressible at levels that are higher than wild-type expression levels;
 - b) combining said plant cell with a compound to be tested for the ability to inhibit the ENR-A, CBL, UROD, PBGD, or CPPO activity under conditions conducive to such inhibition;
 - c) measuring plant cell growth under the conditions of step (b);

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- d) comparing the growth of said plant cell with the growth of a plant cell having unaltered ENR-A, CBL, UROD, PBGD, or CPPO activity under identical conditions; and
- e) selecting said compound that inhibits plant cell growth in step (d).

31. A compound having herbicidal activity identifiable according to the process of claim 30.

- 1 -

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act cat tct acc gtt aac aat acc act gat agc tta aat act atg aat Thr His Ser Thr Val Asn Asn Thr Thr Asp Ser Leu Asn Thr Met Asn 65 70 75 80			240
atc aaa gaa gaa gct agc gtc tct aca tta ttg gtg aac ttg gat aat Ile Lys Glu Glu Ala Ser Val Ser Thr Leu Leu Val Asn Leu Asp Asn 85 90 95			288
aaa ttt gat ccc ttt gat gca atg agc act cog ctt tac caa act gct Lys Phe Asp Pro Phe Asp Ala Met Ser Thr Pro Leu Tyr Gln Thr Ala 100 105 110			336
act ttt aag cag cct tct gct att gaa aat gga cct tat gat tat aca Thr Phe Lys Gln Pro Ser Ala Ile Glu Asn Gly Pro Tyr Asp Tyr Thr 115 120 125			384
aga agt ggc aat cct aca cgg gat gca ttg gaa agc ctc ctt gcg aag Arg Ser Gly Asn Pro Thr Arg Asp Ala Leu Glu Ser Leu Leu Ala Lys 130 135 140			432
ctt gac aag gca gat aga gca ttt tgc ttt act agc gga atg gct gct Leu Asp Lys Ala Asp Arg Ala Phe Cys Phe Thr Ser Gly Met Ala Ala 145 150 155 160			480
ctt agt gct gtt aca cat ctt atc aaa aat ggc gaa gaa att gtt gct Leu Ser Ala Val Thr His Leu Ile Lys Asn Gly Glu Glu Ile Val Ala 165 170 175			528
gga gat gat gta tat ggt ggc tct gac aga tta cta tcc caa gtt gtt Gly Asp Asp Val Tyr Gly Gly Ser Asp Arg Leu Leu Ser Gln Val Val 180 185 190			576
cca aga tct ggc gtt gtg gta aaa cga gta aac aca act aag tta gac Pro Arg Ser Gly Val Val Lys Arg Val Asn Thr Thr Lys Leu Asp 195 200 205			624
gag gtt gct gca att ggt ccc caa aca aag ctt gtg tgg ctt gag Glu Val Ala Ala Ala Ile Gly Pro Gln Thr Lys Leu Val Trp Leu Glu 210 215 220			672
tct cca aca aac cca aga caa caa att tct gat ata cga aaa ata tct Ser Pro Thr Asn Pro Arg Gln Gln Ile Ser Asp Ile Arg Lys Ile Ser 225 230 235 240			720
gag atg gct cat gct caa ggt gct ctt gtg ttg gtg gac aac agt att Glu Met Ala His Ala Gln Gly Ala Leu Val Leu Val Asp Asn Ser Ile 245 250 255			768
atg tca cca gtg ctc tct ogg cca tta gaa ctt gga gct gac atc gtg			816

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Met Ser Pro Val Leu Ser Arg Pro Leu Glu Leu Gly Ala Asp Ile Val		
260	265	270
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Met His Ser Ala Thr Lys Phe Ile Ala Gly His Ser Asp Val Met Ala		
275	280	285
ggt gtg ctt gct gta aaa ggc gaa aaa ttg gca aag gag gtg tat ttc		912
Gly Val Leu Ala Val Lys Gly Glu Lys Ieu Ala Lys Glu Val Tyr Phe		
290	295	300
ctc caa aac tca gaa ggt tct gga tta gct cct ttc gac tgt tgg ctt		960
Leu Gln Asn Ser Glu Gly Ser Gly Leu Ala Pro Phe Asp Cys Trp Leu		
305	310	315
tgc ctt cga gga atc aag aca atg gct tta cgg ata gaa aag caa cag		1008
Cys Leu Arg Gly Ile Lys Thr Met Ala Leu Arg Ile Glu Lys Gln Gln		
325	330	335
gaa aac gca cgg aaa att gca atg tac ttg tct tct cat cca aga gta		1056
Glu Asn Ala Arg Lys Ile Ala Met Tyr Leu Ser Ser His Pro Arg Val		
340	345	350
aag aaa gtg tac tat gct ggt cta cca gat cat cct ggt cac cat ctc		1104
Lys Lys Val Tyr Tyr Ala Gly Ieu Pro Asp His Pro Gly His His Leu		
355	360	365
cac ttc tct cag gcg aag ggt gca gga tca gtt ttt agc ttt ata act		1152
His Phe Ser Gln Ala Lys Gly Ala Gly Ser Val Phe Ser Phe Ile Thr		
370	375	380
gga tca gtt gcg ctt tca aag cat ctc gta gaa acc acc aaa tac ttc		1200
Gly Ser Val Ala Leu Ser Lys His Leu Val Glu Thr Thr Lys Tyr Phe		
385	390	395
400		
agc ata gct gtc agt ttt ggg agt gtt aag tca ctg ata agc atg cca		1248
Ser Ile Ala Val Ser Phe Gly Ser Val Lys Ser Ieu Ile Ser Met Pro		
405	410	415
tgc ttc atg tca cat gca agc ata cct gca gaa gtt cgt gag gcc aga		1296
Cys Phe Met Ser His Ala Ser Ile Pro Ala Glu Val Arg Glu Ala Arg		
420	425	430
ggt ttg acg gaa gat ctt gtc cgt ata tct gca gga att gaa gat gtt		1344
Gly Leu Thr Glu Asp Leu Val Arg Ile Ser Ala Gly Ile Glu Asp Val		
435	440	445
450		
gat gat ttg ata tct gat ctt gac att gcc ttc aaa acc ttc cct ctc		1392
Asp Asp Leu Ile Ser Asp Leu Asp Ile Ala Phe Lys Thr Phe Pro Leu		
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465		

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35 40 45
Asn Ser Phe Lys Leu Asn Cys Val Met Glu Lys Ser Val Asp Gly Gln
50 55 60
Thr His Ser Thr Val Asn Asn Thr Thr Asp Ser Leu Asn Thr Met Asn
65 70 75 80
Ile Lys Glu Glu Ala Ser Val Ser Thr Leu Leu Val Asn Leu Asp Asn
85 90 95
Lys Phe Asp Pro Phe Asp Ala Met Ser Thr Pro Leu Tyr Gln Thr Ala
100 105 110
Thr Phe Lys Gln Pro Ser Ala Ile Glu Asn Gly Pro Tyr Asp Tyr Thr
115 120 125
Arg Ser Gly Asn Pro Thr Arg Asp Ala Leu Glu Ser Leu Leu Ala Lys
130 135 140
Leu Asp Lys Ala Asp Arg Ala Phe Cys Phe Thr Ser Gly Met Ala Ala
145 150 155 160
Leu Ser Ala Val Thr His Leu Ile Lys Asn Gly Glu Glu Ile Val Ala
165 170 175
Gly Asp Asp Val Tyr Gly Gly Ser Asp Arg Leu Leu Ser Gln Val Val
180 185 190
Pro Arg Ser Gly Val Val Val Lys Arg Val Asn Thr Thr Lys Leu Asp
195 200 205
Glu Val Ala Ala Ala Ile Gly Pro Gln Thr Lys Leu Val Trp Leu Glu
210 215 220
Ser Pro Thr Asn Pro Arg Gln Gln Ile Ser Asp Ile Arg Lys Ile Ser
225 230 235 240
Glu Met Ala His Ala Gln Gly Ala Leu Val Leu Val Asp Asn Ser Ile
245 250 255
Met Ser Pro Val Leu Ser Arg Pro Leu Glu Leu Gly Ala Asp Ile Val
260 265 270
Met His Ser Ala Thr Lys Phe Ile Ala Gly His Ser Asp Val Met Ala
275 280 285
Gly Val Leu Ala Val Lys Gly Glu Lys Leu Ala Lys Glu Val Tyr Phe
290 295 300
Leu Gln Asn Ser Glu Gly Ser Gly Leu Ala Pro Phe Asp Cys Trp Leu
305 310 315 320
Cys Leu Arg Gly Ile Lys Thr Met Ala Leu Arg Ile Glu Lys Gln Gln
325 330 335
Glu Asn Ala Arg Lys Ile Ala Met Tyr Leu Ser Ser His Pro Arg Val
340 345 350
Lys Lys Val Tyr Tyr Ala Gly Leu Pro Asp His Pro Gly His His Leu
355 360 365
His Phe Ser Gln Ala Lys Gly Ala Gly Ser Val Phe Ser Phe Ile Thr
370 375 380

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Gly Ser Val Ala Leu Ser Lys His Leu Val Glu Thr Thr Lys Tyr Phe
 385 390 395 400
 Ser Ile Ala Val Ser Phe Gly Ser Val Lys Ser Leu Ile Ser Met Pro
 405 410 415
 Cys Phe Met Ser His Ala Ser Ile Pro Ala Glu Val Arg Glu Ala Arg
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Leu Ser Ile Ser Pro Arg Lys Ser Ile Ser Ser Thr Lys Ser Cys Arg		
20 25 30		
ata gtt cga tgt tcc gtc gag gga act act gta acc gag aga aaa gtc		144
Ile Val Arg Cys Ser Val Glu Gly Thr Thr Val Thr Glu Arg Lys Val		
35 40 45		
tcg gca acc agc gag cca ctt ctt ctg aga gct gtt aaa ggt gaa gtt		192
Ser Ala Thr Ser Glu Pro Leu Leu Arg Ala Val Lys Gly Glu Val		
50 55 60		
gtt gat aga cct ccg gtt tgg ctt atg agg caa gct ggg agg tac atg		240
Val Asp Arg Pro Pro Val Trp Leu Met Arg Gln Ala Gly Arg Tyr Met		
65 70 75 80		
aag agt tat caa act ctc tgt gag aag tat cct tct ttc aga gat aga		288
Lys Ser Tyr Gln Thr Leu Cys Glu Lys Tyr Pro Ser Phe Arg Asp Arg		
85 90 95		
tca gag aat gca gat ctt gtg gtg gaa att tct ttg cag cca tgg aag		336
Ser Glu Asn Ala Asp Leu Val Val Glu Ile Ser Leu Gln Pro Trp Lys		
100 105 110		
gtg ttt aag cca gat ggg gtg att ctg ttc tca gac att ctc act cca		384
Val Phe Lys Pro Asp Gly Val Ile Leu Phe Ser Asp Ile Leu Thr Pro		
115 120 125		

- 9 -

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aga tta aga aat gag gtg aac aat gaa gcc gct gtt ctg gga ttt gtt Arg Leu Arg Asn Glu Val Asn Asn Glu Ala Ala Val Leu Gly Phe Val 180 185 190	576
gga gct cca ttt aca ctt tct tcg tat gta atc gaa ggt ggc tca tct Gly Ala Pro Phe Thr Leu Ser Ser Tyr Val Ile Glu Gly Gly Ser Ser 195 200 205	624
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cta cat gcc tta ctc cag aag ttc aca acc tcg atg ata acg tac ata Leu His Ala Leu Leu Gln Lys Phe Thr Thr Ser Met Ile Thr Tyr Ile 225 230 235 240	720
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gtt gat ccg gga gtt cta ttt gga tcg aaa gaa ttt atc aca agc cgg Val Asp Pro Gly Val Leu Phe Gly Ser Lys Glu Phe Ile Thr Ser Arg 340 345 350	1056

- 10 -

att cat gat act gtg aag aaa gct ggg aga gat aaa cac att ctc aac 1104
Ile His Asp Thr Val Lys Lys Ala Gly Arg Asp Lys His Ile Leu Asn
355 360 365

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 35 40 45
 Ser Ala Thr Ser Glu Pro Leu Leu Arg Ala Val Lys Gly Glu Val
 50 55 60
 Val Asp Arg Pro Pro Val Trp Leu Met Arg Gln Ala Gly Arg Tyr Met
 65 70 75 80
 Lys Ser Tyr Gln Thr Leu Cys Glu Lys Tyr Pro Ser Phe Arg Asp Arg
 85 90 95
 Ser Glu Asn Ala Asp Leu Val Val Glu Ile Ser Leu Gln Pro Trp Lys
 100 105 110
 Val Phe Lys Pro Asp Gly Val Ile Leu Phe Ser Asp Ile Leu Thr Pro
 115 120 125
 Leu Ser Gly Met Asn Ile Pro Phe Asp Ile Val Lys Gly Lys Gly Pro
 130 135 140
 Ile Ile Phe Asn Pro Pro Gln Ser Ala Ala Asp Val Ala Gln Val Arg
 145 150 155 160
 Glu Phe Val Pro Glu Glu Ser Val Pro Tyr Val Gly Glu Ala Leu Arg
 165 170 175
 Arg Leu Arg Asn Glu Val Asn Asn Glu Ala Ala Val Leu Gly Phe Val
 180 185 190
 Gly Ala Pro Phe Thr Leu Ser Ser Tyr Val Ile Glu Gly Gly Ser Ser
 195 200 205
 Lys Asn Phe Thr Gln Ile Lys Arg Leu Ala Phe Ser Gln Pro Lys Val
 210 215 220
 Leu His Ala Leu Leu Gln Lys Phe Thr Thr Ser Met Ile Thr Tyr Ile
 225 230 235 240
 Arg Tyr Gln Ala Asp Ser Gly Ala Gln Ala Val Gln Ile Phe Asp Ser
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 260 265 270
 Tyr Leu Lys Gln Ile Val Glu Ala Val Lys Gln Thr His Pro Asn Leu

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Ala Arg Thr Gly Val Asp Val	Val Ser Leu Asp	Trp Thr Val Asp Met
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	325	330
Val Asp Pro Gly Val Leu Phe	Gly Ser Lys Glu Phe	Ile Thr Ser Arg
	340	345
Ile His Asp Thr Val Lys Lys	Ala Gly Arg Asp Lys	His Ile Leu Asn
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- 12 -

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aat ggt cat att gac ata gct gtg cac tca atg aaa gat gtc cca act Asn Gly His Ile Asp Ile Ala Val His Ser Met Lys Asp Val Pro Thr 145 150 155 160	480
tac tta cca gaa aaa acg att tta cct tgt aac ctt ccg cgt gag gat Tyr Leu Pro Glu Lys Thr Ile Leu Pro Cys Asn Leu Pro Arg Glu Asp 165 170 175	528
gtt cga gat gcg ttt att tgt cta act gca gcc acg tta gct gag ctt Val Arg Asp Ala Phe Ile Cys Leu Thr Ala Ala Thr Leu Ala Glu Leu 180 185 190	576
cca gct gga agc gtt gtg gga aca gct tct ctc agg aga aaa tcg cag Pro Ala Gly Ser Val Val Gly Thr Ala Ser Leu Arg Arg Lys Ser Gln 195 200 205	624
att ctc cac aaa tat cct gca tta cat gtt gag gaa aac ttc agg ggt Ile Leu His Lys Tyr Pro Ala Leu His Val Glu Glu Asn Phe Arg Gly 210 215 220	672
aat gtg cag aca aga cta tca aaa cta caa gga gga aag gtccaa gca Asn Val Gln Thr Arg Leu Ser Lys Leu Gln Gly Gly Lys Val Gln Ala 225 230 235 240	720
act cta tta gca cta gct ggt ctt aag aga ttg agt atg aca gag aat Thr Leu Leu Ala Leu Ala Gly Leu Lys Arg Leu Ser Met Thr Glu Asn 245 250 255	768
gtc gca tct atc tta tct ctc gat gaa atg ctt cca gct gtt gct caa Val Ala Ser Ile Leu Ser Leu Asp Glu Met Leu Pro Ala Val Ala Gln 260 265 270	816
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325

330

335

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 340 345 350

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 Gly Pro Tyr Val Tyr Glu Asp Met Val Lys Met Gly Lys Asp Ala Gly
 355 360 365

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 35 40 45
 Lys Cys Arg Arg Lys Gln Ser Ser Ser Gly Phe Val Lys Ala Cys Val
 50 55 60
 Ala Val Glu Gln Lys Thr Arg Thr Ala Ile Ile Arg Ile Gly Thr Arg
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 Gly Ser Pro Leu Ala Leu Ala Gln Ala Tyr Glu Thr Arg Glu Lys Leu
 85 90 95
 Lys Lys Lys His Pro Glu Leu Val Glu Asp Gly Ala Ile His Ile Glu
 100 105 110
 Ile Ile Lys Thr Thr Gly Asp Lys Ile Leu Ser Gln Pro Leu Ala Asp
 115 120 125
 Ile Gly Gly Lys Gly Leu Phe Thr Lys Glu Ile Asp Glu Ala Leu Ile
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 Asn Gly His Ile Asp Ile Ala Val His Ser Met Lys Asp Val Pro Thr
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 Tyr Leu Pro Glu Lys Thr Ile Leu Pro Cys Asn Leu Pro Arg Glu Asp
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 Pro Ala Gly Ser Val Val Gly Thr Ala Ser Leu Arg Arg Lys Ser Gln
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 Ile Leu His Lys Tyr Pro Ala His Val Glu Glu Asn Phe Arg Gly
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 225 230 235 240
 Thr Leu Leu Ala Leu Ala Gly Leu Lys Arg Leu Ser Met Thr Glu Asn
 245 250 255
 Val Ala Ser Ile Leu Ser Leu Asp Glu Met Leu Pro Ala Val Ala Gln
 260 265 270

- 14 -

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							305		310				315		320
Ala	Gly	Tyr	Ala	Ser	Lys	Asp	Glu	Glu	Gly	Asn	Cys	Ile	Phe	Arg	Gly
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Phe Ser Ser His Arg Leu His Tyr Ser Pro Asn Pro Ser Thr Leu Arg		
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 65 70 75 80

agg gct cgt ttc gag act atg att agg gct gct caa gac agt gtt tgt 288
Arg Ala Arg Phe Thr Met Ile Arg Ala Ala Gln Asp Ser Val Cys
85 90 95

gat gca att gaa gct ata gaa ggc ggt cct aag ttt aaa gaa gat gtt 336
 Asp Ala Ile Glu Ala Ile Glu Gly Gly Pro Lys Phe Lys Glu Asp Val
 100 105 110

- 15 -

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gtt atg cct cct gaa gct tat aga gct gca aag ggc tca gct tct gat Val Met Pro Pro Glu Ala Tyr Arg Ala Ala Lys Gly Ser Ala Ser Asp 145 150 155 160	480
cag aaa cct ggt cct gtt ccg ttc gtc gct gct gga gtc agc tcg gtt Gln Lys Pro Gly Pro Val Pro Phe Phe Ala Ala Gly Val Ser Ser Val 165 170 175	528
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tat ttc gag aca gat gct cca aag gat gtt cct gga gct cca agg caa Tyr Phe Glu Thr Asp Ala Pro Lys Asp Val Pro Gly Ala Pro Arg Gln 195 200 205	624
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ttt gac cct tcc ttc tat ccc cga ttc aag aag tgg tgt gat gac tac Phe Asp Pro Ser Phe Tyr Pro Arg Phe Lys Lys Trp Cys Asp Asp Tyr 245 250 255	768
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acg aca ttt ggt ctg aag aca gga gga cga ata gag agc att ctc gtc		1056
Thr Thr Phe Gly Leu Lys Thr Gly Gly Arg Ile Glu Ser Ile Leu Val		
340	345	350
tct ctt ccg ctt tca gca aga tgg gaa tat gac cat aaa ccg gaa gag		1104
Ser Leu Pro Leu Ser Ala Arg Trp Glu Tyr Asp His Lys Pro Glu Glu		
355	360	365
ggg acc gaa gag tgg aag cwa ttg gat gct tgc atc aac ccg aag gag		1152
Gly Thr Glu Glu Trp Lys Xaa Leu Asp Ala Cys Ile Asn Pro Lys Glu		
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tgg atc tag		1161
Trp Ile		
385		

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<213> Arabidopsis thaliana

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Val Ser Ile Glu Lys Glu Val Pro Glu Thr Glu Arg Pro Phe Thr Phe		
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65 70 75 80		
Arg Ala Arg Phe Glu Thr Met Ile Arg Ala Ala Gln Asp Ser Val Cys		
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Asp Ala Ile Glu Ala Ile Glu Gly Gly Pro Lys Phe Lys Glu Asp Val		
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Trp Ser Arg Pro Gly Gly Gly Gly Ile Ser Arg Val Leu Gln Asp		
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130 135 140		
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145 150 155 160		
Gln Lys Pro Gly Pro Val Pro Phe Phe Ala Ala Gly Val Ser Ser Val		
165 170 175		
Leu His Pro Lys Asn Pro Phe Ala Pro Thr Leu His Phe Asn Tyr Arg		
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Tyr Phe Glu Thr Asp Ala Pro Lys Asp Val Pro Gly Ala Pro Arg Gln		
195 200 205		
Trp Trp Phe Gly Gly Thr Asp Phe Thr Pro Ala Tyr Ile Phe Glu		
210 215 220		
Glu Asp Val Lys His Phe His Ser Ile Gln Lys Gln Ala Cys Asp Lys		
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Phe Asp Pro Ser Phe Tyr Pro Arg Phe Lys Lys Trp Cys Asp Asp Tyr		

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245	250	255	
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Thr Glu Cys Ala Asn Ser Val Val Pro Ala Tyr Ile Pro	Ile Val Glu		
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Lys Arg Lys Asp Met Glu Phe Thr Glu Gln His Lys Ala	Trp Gln Gln		
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- 18 -

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<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
oligonucleotide

<400> 12

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29

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<213> Artificial Sequence

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oligonucleotide

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29

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<212> DNA
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<220>
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oligonucleotide

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27

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<220>
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oligonucleotide

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27

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<213> Artificial Sequence

<220>
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<400> 16
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27

- 20 -

<210> 17
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<212> DNA
<213> Artificial Sequence

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43

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oligonucleotide

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24

<210> 21
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- 21 -

<213> *Arabidopsis thaliana*

<400> 21

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<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
oligonucleotide

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30

<210> 23

<211> 33

<212> DNA

- 22 -

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
oligonucleotide

<400> 23

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33

<210> 24

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
oligonucleotide

<400> 24

cccggtatcca tgtcaatcct tcaagtc

27

<210> 25

<211> 27

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:
oligonucleotide

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27

<210> 26

<211> 3422

<212> DNA

<213> Arabidopsis thaliana

<400> 26

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- 23 -

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